Immunological Markers of Responsiveness against *Teladorsagia* Infections in Sheep

Jennifer Jane Pettit

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Edinburgh

June 2003

Abstract

Gastrointestinal nematode infections are a major source of economic loss in small ruminants, causing ill health; poor weight gain and reduced fibre quality/yield. In Britain, the principal nematode genera associated with disease are *Teladorsagia*, *Nematodirus*, and *Trichostrongylus*. Endoparasitic diseases have, until now largely been controlled using chemotherapy, however the increasing prevalence of anthelmintic resistance has focussed attention upon methods of controlling nematodes which are not solely reliant upon anthelmintics. Such approaches either seek to regulate the free-living population of infective larvae on the pasture (e.g grazing management or by using nematophagous fungi) or regulate the parasitic population by enhancing the expression of host immunity (e.g. vaccination, improved host nutrition, and genetic selection).

Studies to define the mechanisms of immunity to gastrointestinal nematodes have strongly implicated Th_2 responses, involving the influx/proliferation of inflammatory cells into the local mucosae, and the generation of IgE antibody. Previous investigations have indicated that IgE antibody may provide a useful marker for immune status. IgE response is directed predominantly against the L_3 stage of *Teladorsagia circumcincta* larvae leading to the recognition of a specific *T.circumcincta* L_3 antigen, which seems to play a vital role in the stimulation of local IgE antibody production.

The main objective of this study was to identify immunological traits, which are associated with resistance and susceptibility, and particularly the relative significance of IgE antibodies in the response to gastrointestinal nematodes in sheep. 80 Greyface Suffolk Cross lambs that were grazing on pasture infected with *T.circumcincta* were ranked in order of their cumulative resistance to nematode infection as determined by their individual faecal egg counts from material taken from the middle, the end and the overall grazing season. The 10 lambs that had the lowest egg count rankings termed "responders" and 10 lambs that had the highest egg count rankings termed "non-responders" were selected for each of the three time periods. Serum samples collected from these lambs during the grazing season were tested on ELISA, using the specific *T.circumcincta* L_3 antigen to detect concentrations of both IgE and IgA antibodies. Results showed that the responder lambs selected from the middle and overall grazing season showed significantly higher serum IgE antibody titres ($p<0.05$) than the non-responders towards the end of the season. No differences in IgA levels were observed between responders and non-responders at any time period. A two-

year study was then carried out to examine if any other relationships exist between responsiveness against gastrointestinal nematodes in lambs and other immunological traits.

98 Scottish Blackface lambs that were grazing on pastures naturally infected with *T.circumcincta* were ranked in order of their responsiveness to nematode infection as described previously and 10 responders and 10 non-responders for each time period were selected. Six factors were investigated from the lambs including IgE, IgA, IgG antibody titres, eosinophils, weight gain and numbers of IgE bearing cells. The study was followed for two grazing seasons. Results showed that in the first grazing season the most important markers of responsiveness to nematodes appeared to be the eosinophils and the numbers of IgE bearing cells. The second grazing season showed that the main marker of responsiveness seemed primarily to be IgE bearing cells, but no correlation was determined for circulating IgE antibody. This latter study indicates that IgE bearing cells may provide a useful marker for selecting animals that are responsive against *T.circumcincta* infections in lambs.

Declaration

The work presented in this thesis was carried out at the Moredun Research Institute, Edinburgh. The experimental work and the interpretation of the results were undertaken by the author. Contributions to the work in this thesis by colleagues are fully acknowledged in the text.

This work has not been nor is currently being submitted for candidature for any other degree.

Jennifer Jane Pettit
Moredun Research Institute
Edinburgh
June 2003

Acknowledgements

I would like to thank the following people for all their help and encouragement during the preparation of this thesis:

My supervisors at Moredun Research Institute, Dr Frank Jackson and Dr John Huntley, for all their enthusiasm, patience and support.
My university supervisor, Dr David Collie, for regular helpful advice and discussion.
Liz Jackson and the Parasitology staff for all their help, both on farm visits and in the laboratory.
Dr Mara Rocchi for advice and assistance with flow cytometry.
Kevin McLean and Daniel Pettit for their help and support with mass spectrometry and N-terminal amino acid sequencing techniques.
Jim Redmond, Annie MacKellar and Judith Machell for all their technical skills.
Jill Sales for her guidance with the statistical analyses.
The Meat and Livestock Commission for funding this project.

I am very grateful for the support and encouragement of my family and friends over the course of this study.

This work is in memory of my mum, Alison and my Grandfather, Jack who gave me the utmost support for this project but sadly passed away before it was complete.

TABLE OF CONTENTS

CHAPTER 2 GENERAL MATERIALS AND METHODS

CHAPTER 3 PURIFICATION AND CHARACTERISATION OF *TELADORSAGIA CIRCUMCINCTA* THIRD STAGE (L_3) LARVAL ANTIGEN

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Abbreviations

Abbreviations

ρ	rho, Spearman's correlation
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
BZ	Benzimidazole
CD4	Cluster of differentiation 4
CD23	Cluster of differentiation 23
CO	Combination Benzimidazole and Ivermectin
C.curticei	*Cooperia curticei*
dH_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Epg	Eggs per gram
ES	Excretory/Secretory
FACS	Flow Activated Cell Sorter
FcεRI	High affinity receptor
FcεRII	Low affinity receptor
FEC	Faecal Egg Count
g	gravity
γδ T cell	gamma delta T cell
HCl	Hydrochloric acid
H.contortus	*Haemonchus contortus*
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL	Interleukin
IVM	Ivermectin
LEV	Levamisole
kDa	Kilo Daltons
Kg	Kilogram
l	Litre
LC	Liquid Chromatography
L_1	First stage larvae
L_2	Second stage larvae
L_3	Third stage larvae
L_4	Fourth stage larvae
L_5	Fifth stage larvae
Mab	Monoclonal Antibody
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation – Time Of Flight
ml	Millilitre
mM	Millimolar
MS	Mass Spectrometry
MMC	Mucosal mast cells
MUMB	Medicated Urea Molasses Blocks
$NaHCO_3$	Sodium Hydrogen Carbonate
NaCl	Sodium Chloride
NaN_2	Sodium Azide
N.battus	*Nematodirus battus*
N.brasiliensis	*Nippostrongylus brasiliensis*
OD	Optical Density
OPD	Ortho-phenylenediamine
O.ostertagi	*Ostertagia ostertagi*
°C	degrees Celsius
PBS	Phosphate Buffered Saline
PPR	Periparturient rise
PPRI	Periparturient Relaxation in Immunity
RE	Rapid Expulsion

Rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SRS-A	Slow reacting substance of anaphylaxis
T20	Tween 20
T80	Tween 80
T.axei	*Trichostrongylus axei*
TEMED	N,N,N',N'-Tetramethylethylenediamine
T.circumcincta	*Teladorsagia circumcincta*
T.colubriformis	*Trichostrongylus colubriformis*
T.spiralis	*Trichinella spiralis*
Th1	T helper 1
Th2	T helper 2
TNF-α	Tumor Necrosis Factor alpha
μg	Microgram
μl	Microlitre
UMB	Urea Molasses Blocks

Chapter 1
General Introduction

1.1 NATURAL HISTORY OF NEMATODES

The phylum Nemathelminths have six classes, yet only one of these contains worms of parasitic importance. These are the nematoda (Urquhart, *et al* 1991).
The nematoda belong to a group of metazoan parasites collectively termed as the helminths. They are a ubiquitous and successful group of parasites found in marine, fresh water and terrestrial habitats and parasitise plants and animals.

In the nematoda, the sexes are usually separate but in some species where males have not been found, reproduction is parthogenetic (Urquhart *et al.*, 1991). Nematodes are either oviparous or ovoviviparous (forming eggs that develop and hatch whilst in the body of the female). The larvae are similar in appearance to adult worms but lack gonads (Urquhart *et al*, 1991).

Nematodes cause a variety of disease syndromes in animals, the consequences of which are of particular economic importance to agricultural production worldwide. Parasitic nematodes are classified into six orders, one of which the strongylida contains the principal gastrointestinal nematodes termed the Trichostrongyles.

1.2 THE LIFE CYCLE OF THE TRICHOSTRONGYLES

This group of parasites exist as separate sexes, the males being generally smaller than the females. They have a direct life cycle with no intermediate host (Urquhart *et al.*, 1991). During development the nematode moults at intervals, shedding its cuticle. In the complete life cycle there are four stages, designated L_1 to L_4, an immature adult (L_5) and an adult stage. The life cycle of the trichostrongyles is illustrated in figure 1.1.

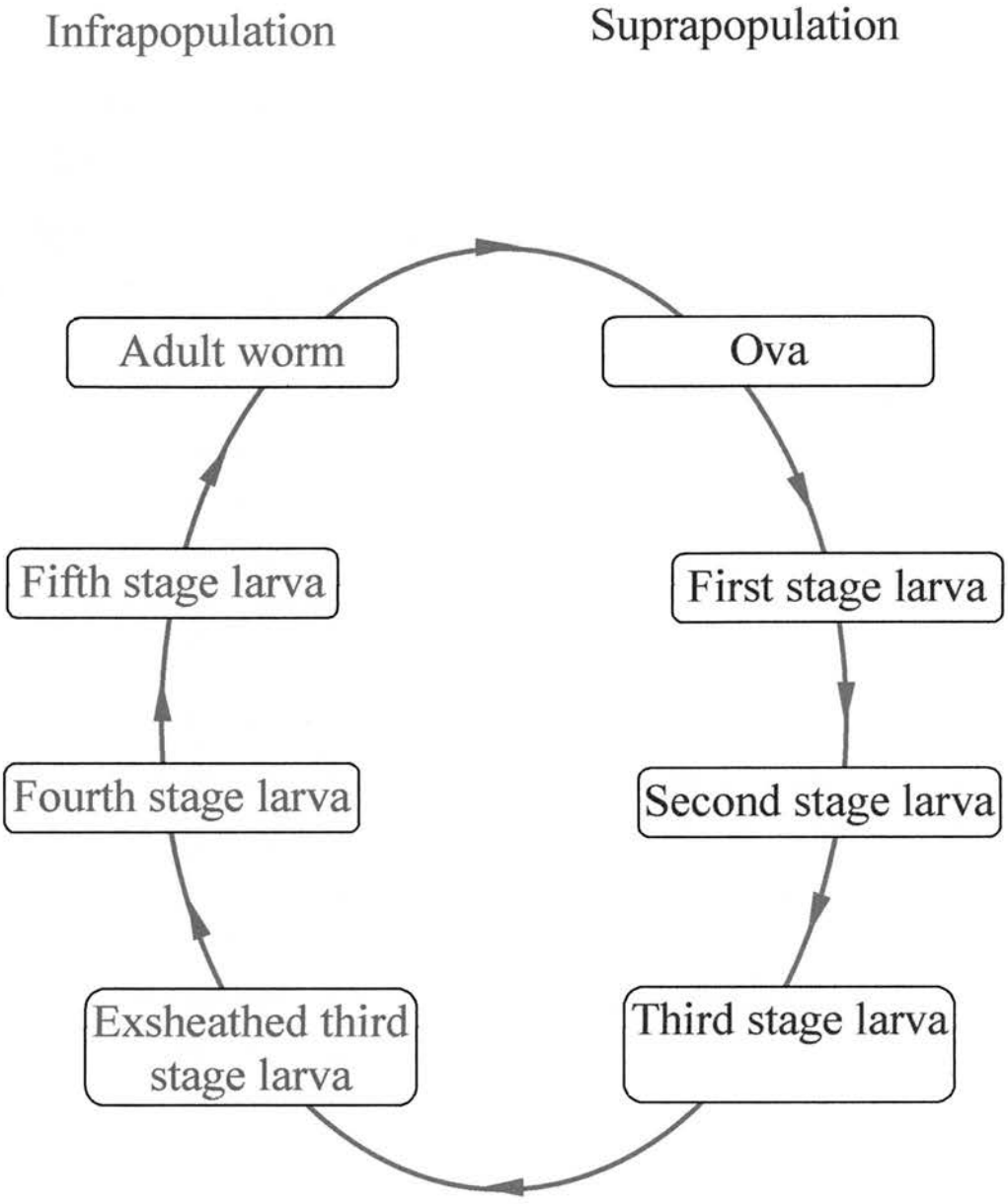


Figure 1.1 Life cycle of the Trichostrongyles

In the common form of the direct life cycle, the L_1 to L_3 larvae are free living whilst the L_3 to L_5 larvae and adult worms live within the host's gastrointestinal tract, intimately associated with the mucosa. The adult parasites derive nourishment by either browsing on the gut mucosal surface, or by imbibing host blood components. After copulation, the adult female lays a substantial number of eggs, which are subsequently voided onto the pasture with the host's faeces. Given suitable temperature and moisture conditions egg development occurs and free living first stage larvae hatch. The L_1 larvae feed on faecal material and following two moults become the infective third stage larvae. The second moult is incomplete, a new cuticle being secreted underneath the old one, which is retained as a protective sheath. The ensheathed larva is a non-feeding stage, infective to host animals, and can remain viable in large numbers on the pasture for some months, depending on environmental conditions. Since the third stage larva cannot feed, it relies upon fat and reserves of glycogen accumulated during earlier larval stages until it is ingested by an appropriate host. Once ingested, the larva is stimulated to exsheath in the stomach or intestine and usually penetrates the mucosa where it moults twice before emerging as an adult.

The trichostrongylids are responsible for considerable mortality and widespread morbidity, especially in ruminants. The most significant gastrointestinal parasites of small ruminants are demonstrated in table 1.1.

Location	Scientific Name	Common Name
Abomasum	*Teladorsagia circumcincta* (*T.circumcincta*)	Small brown stomach worm
	Haemonchus contortus (*H.contortus*)	Barber's pole worm
	Trichostrongylus axei (*T.axei*)	Stomach hair worm
Small Intestine	*Cooperia curticei* (*C.curticei*)	Cooperia spp.
	Nematodirus battus (*N.battus*)	Nematodirus spp.
	Trichostrongylus vitrinis (*T.vitrinis)*	Trichostrongylus spp.

Table 1.1 Significant gastrointestinal parasites of small ruminants

1.3 EPIDEMIOLOGY

The normal parasitic life cycle of nematodes can be affected by two phenomena that are of significant biological and epidemiological importance. These are arrested larval development and the periparturient relaxation in immunity.

1.3.1 Arrested Larval Development

Arrested development is a significant feature of the life cycle of a number of nematode species. It is of particular importance in the trichostrongyles of grazing ruminants. The process of arrested development also known as hypobiosis may be defined as a temporary halt in the parasitic phase of development at a specific point in the nematode life cycle (Michel, 1974).

Arrested larvae not only stop growing but their metabolic rate also decreases significantly and they stop moving. They can survive in this state for weeks or months before resuming development. A change in the environment is the most common

external stimulus that leads to the hypobiosis of nematodes in the host. Thus the accumulation of arrested larvae is often linked with the start of cold winter conditions in the northern hemisphere, or very arid conditions in the sub tropics or tropics (Urquhart *et al*, 1991). However, these arrested larvae soon begin to grow and mature with the return of suitable environmental conditions to their free-living development (Urquhart *et al*, 1991). Arrested larval development is also caused by the high density of adult worms causing the "feedback" inhibition of incoming infective larvae which go into arrest until the adult worm population decreases in number or is eliminated. Another factor responsible for hypobiosis is the host immune response inhibiting the normal development of the parasitic stage of the life cycle (Gibbs, 1986).

The epidemiological importance of arrested larval development is that even during periods of adversity, the survival of the nematode is maintained. Also, the resumption of development and maturation of arrested larvae increases the contamination of the environment and can subsequently result in outbreaks of clinical disease (Urquhart *et al*, 1991).

1.3.2 The Periparturient Relaxation in Immunity (PPRI)

A periparturient relaxation in immunity during pregnancy and lactation has been reported in a variety of species including rabbits (Dunsmore, 1966), sheep (Connan, 1968), rats (Connan, 1972), mice (Ngwenya, 1977) and more recently goats (Rahman & Collins, 1992). In view of the epidemiology of infection it is essential to take into account the increase in faecal egg counts (FECs) observed as a consequence of this PPRI. The periparturient rise (PPR) in FEC in ewes remains a poorly understood phenomenon. The sheep nematodes that are involved in causing this PPR in FEC in the United Kingdom are predominantly the abomasal parasites, *H.contortus* and *T.circumcincta*. Resistance to the establishment of incoming larvae, constraints on the fecundity of female worms and the capacity to expel adult worms are all diminished during the periparturient period (O'Sullivan & Donald, 1970, 1973; Connan, 1968; Michel *et al*, 1979; Donald *et al*, 1982). Investigations mainly with sheep have

demonstrated that the PPR in faecal egg count is associated with lactation and is probably related to a temporary depression of the immune response during that period (Connan, 1968). It was initially suggested that changes in the circulating levels of the lactogenic hormone, prolactin, suppresses immunity, allowing a dramatic increase in faecal egg output from approximately two weeks before lambing until eight weeks post-lambing. More recent investigations have examined the theory of a nutritional basis for the PPRI in ewes. The physiological processes that are responsible for the reproductive cycle impose substantial nutrient demands on a female. For example, energy and protein requirements can increase between two- and tenfold during lactation (Blaxter, 1989; Jessop, 1997), levels that are otherwise rarely reached. A number of studies have demonstrated that there may be competition for nutrient supply between the immune system and the reproductive demand. Consequently, it has been suggested that PPRI could be due to reproductive functions taking priority over immune functions for the use of insufficient nutrients. (Houdijk *et al.*, 2001). The lack of nutrients occurs due to an imbalance between nutrient supply and demand. Houdijk *et al.,* (2001) proposed that an increased supply of, or a decreased demand for nutrients, lowers the extent of PPRI.

The epidemiological importance of the PPR is that it occurs at a point when the numbers of new susceptible hosts are increasing and so may enhance the survival and generation of the worm species. Depending on the size of infection, it may also cause a loss of production in lactating animals and by contamination of the environment lead to clinical disease in susceptible young animals.

1.4 PATHOGENESIS

The presence of trichostrongyles within the abomasum and intestine of ruminants may give rise to extensive pathological and biochemical changes with severe clinical consequences.

The developing parasites which target the abomasum (*H.contortus* and *T.circumcincta*) cause a reduction in the functional gastric gland mass responsible for the production of the highly acidic proteolytic gastric juice. These changes eventually

spread to the surrounding non-parasitised glands with the end result being a thickened hyperplastic gastric mucosa (Urquhart *et al*, 1991).

Some trichostrongyles, most notably *H. contortus*, are blood feeders and may remove as much as 0.5ml of blood per day by ingestion and seepage from the lesions with the outcome that a considerable blood loss may be suffered even with a moderate worm burden. The result is haemorrhagic anaemia, the consequences of which are a loss of iron and protein into the gastrointestinal tract along with a dramatic fall in packed red cell volumes (PCV) (Holmes, 1985).

In trichostrongyle infection, clinical signs usually include some or all of the following; inappetance, weight loss, weakness, diarrhea and in severe cases, death. The marked depression in appetite, together with losses of plasma protein into the gastrointestinal tract, result in interference with the post-absorptive metabolism of protein and to a lesser extent the utilisation of metabolisable energy (Urqhuart, 1990). In lambs with moderate infections, carcass evaluation shows poor protein, fat and calcium deposition (Urquhart, 1990). The severity of clinical signs depends on the level of worm burdens, age of sheep and importantly the immunological and nutritional status of the host.

1.5 RESILIENCE AND RESISTANCE OF RUMINANT HOST

Resilience is the ability of an animal to withstand the effects of parasitic infection whereas resistance to gastrointestinal nematodes is the ability of the host to prevent establishment and/or development of parasitic infection.

Resistance and resilience of the ruminant host to gastrointestinal parasitic nematode infections are influenced by many factors including nutrition, age, gender, immune status and genetic make-up of the host. The following will provide a brief outline of the influence of those factors on host resilence and resistance.

1.5.1 Age

Young lambs under six months of age are in general more susceptible to infectious diseases than mature sheep. This susceptibility has been described for a few viral diseases and a number of bacterial infections (Weiss *et al.*, 1986) but is most apparent for infections with gastrointestinal nematodes (Urquhart *et al.*, 1966; Neilson, 1975; Dineen *et al.*, 1978). Several studies have demonstrated that age influences the ability of an animal to acquire resistance to gastrointestinal nematodes. In general, lambs are more susceptible to *H.contortus* than adult sheep and their susceptibility decreases as they grow older, provided they are exposed to parasite antigens. Gibson *et al* (1972) suggested that older lambs infected with *Trichostrongylus colubriformis* (*T.colubriformis*) were able to resist the establishment of infection more efficiently than younger animals. The susceptibility of young ruminants to nematode infections appears to be due mainly to immunological hyporesponsiveness, and it is not simply a consequence of the lambs not having been exposed adequately to pathogens to develop active immunity. The immune system of the lambs appears to develop through a maturation method, which begins during foetal life and continues during the first 12 months of life (Watson & Gill, 1991). Lambs appear to respond to nematode infection with conventional antibody and cell-mediated responses to a variety of antigens in the neonatal period, albeit with a reduced rate and magnitude compared with that in adults (Watson & Gill, 1991;Colditz *et al.*, 1996).

1.5.2 Gender

The gender of the lamb affects the development of the immune response with males having less intense immunological responsiveness than females (Luffau *et al*, 1981). In general, it is understood that male sheep are more susceptible to nematode infections than female sheep (Barger, 1993). This had been shown in experimental infections with *Oesophagostomum columbianum* (Dobson & Bawden, 1974; Bawden, 1969) with *T.colubriformis* (Windon & Dineen, 1980) and with *H.contortus* (Luffau *et al*, 1981). Suggested mechanisms have been investigated by Schuurs & Verheul (1990). They have

reported that circulating levels of major immunoglobulin classes and of antibodies to a large number of antigens are higher and more persistent in females than in males. Equally, cell mediated immune responses are more active in females and castrated males than they are in intact males. Levels of oestrogens in females have been demonstrated to generate antibody and cell mediated immune responses, while androgens have the reverse effect. The specific mechanisms, however, remain undefined. Schuurs & Verheul (1990) suggests that the increased immune response of females compared with males allows them to counteract increased environmental challenges consequent on the stress of reproduction. This may account for their longevity and greater susceptibility to autoimmune disease.

1.5.3 Breed differences

There is considerable interest in the fact that some breeds of domestic ruminants are more susceptible to certain parasitic infections than others. Difference in susceptibility to *H.contortus* between breeds of sheep have been investigated by many workers in different parts of the world. It has been demonstrated that the Red Masai sheep, indigenous to East Africa, are more resistant to an infection of *H.contortus* than several imported breeds studied in that area (Urquhart *et al*, 1991). This is probably partly due to the evolutionary advantage the indigenous breeds have over the imported breeds. Yadav *et al* (1993) suggested that Hisaradale lambs (an exotic cross breed) had a greater susceptibility to *H.contortus* infection than the indigenous cross breeds known as the Munjal lambs. Resistance to *H.contortus* was also greater in Scottish Blackface lambs than Dorset lambs (Altaif & Dargie, 1978). More recently, New Zealand studies have also suggested that Texel crosses may be more resistant to parasitic infection (Douch *et al*, 1996). A reason for some sheep breeds to be more resistant to nematode infection may be due to the animals' differences in the rate of development of immune responsiveness to the parasite infection.

1.5.4 Weaning

Weaning and the absence of the dam's milk is another trait affecting the growth of parasite resistance in lambs (Spedding *et al.*, 1963). Milk is an important source of nutrients and of other growth or immunologically active factors. Watson & Gill (1991) discovered that suckling lambs developed earlier and more elevated serum antibody responses to parasite antigens of *H.contortus* than weaned lambs. Behavioural changes related to weaning and the elimination of the mother's influence on the lamb's choice of diet from the pasture may also affect the metabolic and nutritional status of the lamb (Jordon *et al.*, 1968). Watson & Gill (1991) reported a study whereby weaned and unweaned lambs were infected with 5000 *H.contortus* larvae from 8 weeks of age. They discovered that, by 12 weeks of age, the weaned group had lower PCV and double the mean FEC of the unweaned group of lambs indicating that the weaned lambs had a lower resilience and resistance to the parasites than the unweaned group. Weaning has also been suggested to affect the rate of development of resistance to parasites in other ways. Weaning of young lambs seems to stimulate the secretion of adrenal glucocorticoids (Watson & Gill, 1991), however, there is limited work relating the level of hormone secreted at weaning with dose regimes required to suppress immunity.

1.6 ANTHELMINTICS - A STRATEGY TO CONTROL GASTROINTESTINAL NEMATODES

Anthelmintics are used to control, prevent and treat parasitic infections of humans, companion animals and agricultural livestock. The largest economic market for anthelmintics is for livestock, mostly for ruminants and particularly cattle and sheep. Although helminth diseases have long been recognised as a significant cause of lost production, effective anthelmintics have only been available for the farming community since the 1950's. Anthelmintics will continue to be the main method of prophylactically controlling helminthoses until effective vaccines or alternative methods of control are produced and/or standards of pasture management have been improved (Pritchard,

1993). Since they have the ability to rapidly remove helminth populations, anthelmintics will always be important therapeutically.

There are three groups or families of broad-spectrum anthelmintic drugs currently available which meet many of these criteria. Drugs within each family have the same mode of action, which is different to the mode of action of other families. All of the currently licensed drugs meet the safety and efficacy standards imposed by the registration authorities. These drugs have the common characteristic that they kill the parasites and do not harm the host when the prescribed concentrations are used.

The first groups of broad-spectrum anthelmintic drugs to be developed were the benzimidazole derivatives, which have been marketed extensively since the 1960's. It has been demonstrated from studies on these compounds that benzimidazoles are antimitotics that disrupt the microtubules and mitotic spindles of the parasite, but not that of the host (Lacey, 1988). In addition, they generally act on the intestinal cells of helminths preventing glucose uptake and thus 'starving' the parasite.

Another group of broad-spectrum anthelmintic drugs were developed in the early 1970's and consist of the imidothiazoles (including drugs such as levamisole and tetramisole) and the tetrahydropyrimidines (including drugs such as morantel). These cholinergic agonists cause spastic paralysis of the parasites, which may not be killed immediately but are subsequently expelled by the host (Pritchard *et al.*, 1994).

The third group of broad-spectrum drugs is the macrocyclic lactone family. The first compounds in this family, the avermectins, became available in the 1980's (Campbell, 1993). These drugs are unique in that they are particularly effective against both nematodes and arthropods and consequently are often referred to as endectocides (Shoop *et al*, 1993). The avermectins are a group of macrocyclic lactones derived from the soil microorganism *Streptomyces avermitilis* (Marriner, 1986). Other narrow spectrum drugs are also used as anthelmintics. These include the organophosphates, salicylanilides and piperazines. All of these drugs, however, have limitations with regard to spectrum of activity and environmental effects.

As a result of persistent over use of anthelmintics, resistance by parasites against these drugs has rapidly developed. A number of parasitic species are now resistant to the majority of anthelmintics (Pritchard *et al*, 1994). Anthelmintic resistance may be defined as a "heritable change in the ability of individual parasites to survive the recommended therapeutic dose of an anthelmintic drug" (Taylor and Hunt, 1989). Anthelmintic resistance is usually recognised only when parasite control procedures fail. Throughout the world, resistance to anthelmintic drugs has been described frequently in the gastrointestinal nematodes of sheep and goats, most commonly *H.contortus* and *T.circumcincta* and also to a lesser extent, in parasites belonging to the genera *Trichostrongylus, Cooperia,* and *Nematodirus*. Several studies on anthelmintic resistance have suggested that it is inherited as a polygenic trait (Le Jambre *et al*, 1982). It has also been proposed that anthelmintic resistance in nematodes may be due to specific genes that are already existent in the population at low frequency, before any anthelmintics have been used (Jackson, 1993). When the population is then exposed to anthelmintics, the susceptible worms are killed and the next generation consists of offspring from the resistant minority (Taylor and Hunt, 1989). In this way resistance to a drug may be amplified from one generation to the next.

Most anthelmintics are non-persistent chemicals, which are eventually cleared from the host. It may be that the nematodes able to withstand treatment (i.e. resistant parasites) have the ability to temporarily reduce their energy demands or switch to alternative energy pathways in order to decrease the uptake of anthelmintic. This facility forms the basis for resistance and will be inherited by their offspring (Taylor and Hunt, 1989).

The advent of drug resistance in many countries together with significant public concern over the presence of chemical residues in meat products, suggest that control based on anthelmintics is not sustainable. Several avenues of research are currently being investigated as alternative strategies to tackle the problem of parasitic infection and/or reduce the reliance on anthelmintics.

1.7 PASTURE AND GRAZING MANAGEMENT

In theory, competent pasture management may effectively control nematode parasites. In temperate regions of the world, combining anthelmintic treatment with some form of grazing management is suggested to improve efficiency of parasite control in livestock (Barger, 1987). Michel (1985) has described grazing management systems as being preventive, evasive or diluting. Preventive strategies provide clean pastures for the grazing of animals infected with a small number of parasites. Evasive strategies depend upon the movement of infected stock away from contaminated pastures. Diluting strategies utilise co-grazing resistant stock as a way of reducing the contamination of pastures. The main role of grazing management in nematode control methods based on epidemiological information is to provide clean pastures on which livestock may graze safely (Barger, 1999). The most significant factors in determining the success of integrated grazing programmes are the host specificity of the parasite, the longevity of the free-living stages and the duration of pasture resting or alternative use (Waller, 1997). However, grazing management can be costly in terms of land, fencing and also labour.

1.8 DIETARY SUPPLEMENTATION

The nutritional status of the host and the impact of gastrointestinal infection have been established for a long time, with underfed animals displaying increased susceptibility to parasitism. Clunie-Ross (1932) suggested that loss of production and increased mortality rates associated with parasitic nematode infection may be reduced with an improvement in the nutritional state of the host.

The majority of investigations concentrate on proteins as the major source of nutrition. Coop & Kyriazakis (1999) found that protein content within the ruminant diet, regardless of the quantity of food consumed was associated with susceptibility to parasitic disease. Additional studies have been embarked on by a number of research groups investigating a range of different amounts and types of protein in the diet, for example: low concentration of protein versus high concentration of protein. Abbot *et al*

(1985) demonstrated that lambs infected with *H.contortus* on a low protein diet ($88gkg^{-1}$ dry matter) were less able to resist the pathophysiological consequences of infection than lambs on a high protein diet ($169gkg^{-1}$dry matter). The worm burdens and loss of abomasal blood were comparable, however, the lambs given the low protein diet developed more severe clinical signs that included anorexia. It was therefore evident that the protein metabolism in lambs given a low protein diet was severely affected compared to lambs on a high protein diet, which enhanced the pathogenic effects of *H.contortus* infection in the former group.

Similar diets were then given to lambs presented with a continuous infection of *H.contortus* (as would be the case on pasture). The animals on a low protein diet repeatedly showed more severe clinical signs and pathophysiological changes. In addition, lambs on a high protein diet developed a stronger immunological resistance to further infection than lambs on a low concentration of protein.

A range of other dietary supplements have also been employed in an attempt to combat nematode infection. Dietary proteins fed to ruminants are degraded to various degrees by microorganisms found naturally in the rumen (first stomach) (Kezar & Church, 1979). This is a rather inefficient mechanism and it would be beneficial to prevent this degradation so that useful proteins can pass directly into the abomasum ("true stomach") for utilization by the host in order to boost immunological responsiveness or improve physical tolerance to infection and so reduce clinical symptoms. Such proteins, that are resistant to degradation within the rumen, are termed by-pass proteins.

An experiment conducted by van Houtert *et al* (1995) investigated the influence of fishmeal as a source of rumen by-pass protein upon the immunity of lambs to *T.colubriformis* infection. Results demonstrated that supplementary feeding substantially reduced the production losses attributable to infection with *T. colubriformis* and was associated with enhanced expulsion of the parasite burden.

Another approach towards investigating the effect of by-pass protein supplementation involves administering the protein supplement directly into the

abomasum, thus avoiding degradation by the rumen flora. This is achieved by attaching an abomasal cannula to the lateral wall of the lambs' abomasum and exteriorizing this between the last two ribs. A protein supplement, sodium caseinate, is continuously infused through the cannula. Coop *et al* (1995) used this procedure to examine by-pass protein supplementation on the development of immunity to *T.circumcincta*. A group of lambs fitted with an abomasal catheter were infected daily with 2000 *T.circumcincta* L_3 larvae for eight weeks. Casein solution was infused through the abomasal catheter providing the lambs with 45 grams of crude protein per day. The group of lambs, including a control (non-supplemented) group, were then treated with anthelmintic, challenged one week later with 50,000 *T.circumcincta*, and killed after a further ten days. It was evident that the infusion of casein had improved the development of immunity to *T.circumcincta*. Egg counts of the supplemented lambs were lower compared to the non-supplemented lambs and the supplemented lambs contained fewer challenge worms. This experiment determined that the detrimental effects of the *T.circumcincta* infection were reduced by the daily infusion of sodium caseinate.

Low cost urea supplementation has been investigated in South-East Asia and the Pacific Islands where there is a vital need to improve the nutritional intake of small ruminants parasitised with gastrointestinal nematodes (Knox & Steel, 1996). Knox & Steel (1996) demonstrated that urea supplementation increased the food intake of the sheep and reduced the faecal egg output. However, increased food intake alone would not be adequate to account for the decreased egg count and the more likely explanation is that of improved immunological regulation of the parasite by the host arising from an enhanced nutritional status. Another example of a low cost supplement containing urea which can improve the animals ability to make use of the diet available and help the animal to fight against parasitic infection are urea-molasses blocks (UMB) (Knox & Steel, 1996). Replacing UMB with medicated UMB (MUMB) can be done for a short time when parasite challenge is extreme or during times of low host immunocompetence brought on by immaturity or physiological stresses such as pregnancy. It is therefore suggested that MUMB will pay an essential role in crucial parasite control programmes

in developing nations where UMB offer significant advantages in the nutritional status of small ruminants.

Some studies have investigated the role of minerals on host resilience to gastrointestinal parasitism by small ruminants. For example, van Houtert *et al* (1995) observed a marked improvement in cumulative weight gain in young sheep infected with *Trichostrongylus vitrinus* if they had been given phosphorus supplements in their diet. It should be noted that, although relevant, the effect of trace element/mineral supplementation is only a minor factor concerning the host's resistance to parasitism. However, in countries where there is a distinct absence of certain trace elements, namely developing countries, supplementation may have more importance.

1.9 BIOLOGICAL CONTROL

Biological control may be defined as "an ecological method designed by man to lower a pest or parasite population to acceptable sub-clinical densities or to keep these populations at a non-harmful level using natural living antagonists" (Grønvold *et al*, 1996). Several biological control programmes are available for insect pathogens. One example would be the introduction of the lady beetle into California to control the cottony cushion scale on citrus trees (Flint, 1998).

1.9.1 Fungal Control

The free-living stages of parasitic nematodes can also be regulated by using biological control procedures. This process is exemplified by the incorporation of nematophagous fungal spores into animal foodstuff, which inhibits the growth of larvae. In this process, the fungus *Duddingtonia flagrans* produces a sticky three-dimensional network that significantly reduces the amount of infective trichostrongyle larvae in faeces from spore fed animals (Larsen, 1999). The fungal spores can survive passage through the gastrointestinal tract of the animals and they are afterwards found with the parasite eggs in fresh faeces. They then develop, grow, trap and hence destroy parasitic larvae (Wolstrup *et al*, 1994). Work is now underway to develop practical delivery

systems in order to put this tool into practice in future integrated control strategies (Hertzberg *et al*, 2002). The great potential of this fungus as a biological control agent has been demonstrated in a number of trials with cattle, sheep, horses and pigs (Larsen *et al.*, 1997). These trials however, have also pointed towards some potential limitations in the activity spectrum of the fungus and it is not known if these fungi are able to cope with the hardened shell of, for example, *Ascaris* spp. or *Trichuris* spp. eggs. Investigations are underway to study the effect of fungi on eggs at different developmental stages (Hertzberg *et al.*, 2002). There is also a requirement to measure any potential influence on the environment as well as effects of other biotic factors on the activity and continued existence of the nematode-destroying fungi when used in integrated control strategies (Larsen *et al.*, 1997).

1.9.2 Vaccination

Many attempts have been undertaken over the years to produce commercially viable vaccines against gastrointestinal nematodes. The only nematode vaccines which have been commercialised successfully for ruminants so far are those involving irradiation and consequent attenuation of living bovine lungworm *Dictyocaulus viviparous* and ovine lungworm, *Dictyocaulus filaria* (Dhar & Sharma, 1981). As summarised below, progress towards the development of vaccines against other GI nematodes has been made with highly protective 'hidden antigens', especially for *H.contortus*, and also with new 'natural antigens'. Antigens that are recognised by the host during the course of nematode infection are named "natural" and antigens that are not seen immunologically by the host during infection are termed "hidden" (Smith, 1999).

Natural Antigens

Markedly protective effects have been described against *H.contortus*, utilizing proteins from adult nematode excretory-secretory (ES) products that are seen immunologically by infected sheep. One study demonstrated that vaccination with a 70-83 kDa surface antigen of exsheathed L_3 *H.contortus* produced protection in five-month

old sheep (Jacobs *et al.*, 1999). The protection generated with this natural antigen is dependent on the induction of a Th_2 response (Jacobs *et* al., 1999). Schallig *et al.* (1997) reported that immunisation with purified 15 and 24 kDa E/S products provided significant protection against a challenge infection in older lambs and sheep, with immunity being Th2 associated and involving mastocytosis (Schallig *et al.*, 1997). These E/S products however, did not give protection to young lambs. The above examples of natural antigen preparations are acquired from living parasites. Recombinant expression of these known protective antigens may aid their large-scale production. However, both of these antigen preparations comprise glycosylated proteins (Ashman *et al.*, 1995; Schallig *et al.*, 1997) that may confound production by conventional recombinant DNA technology.

Hidden Antigens

The approach of hidden antigen vaccines has been independently developed for the cattle tick *Boophilus microplus* (Willadsen *et al.*, 1989) and for *H.contortus*, both blood feeders. The foundation of these hidden antigen vaccines is that immunisation of a host with a surface membrane protein from the GI tract of the nematode will result in the production of a high concentration of circulating antibodies to this antigen. When the nematode then imbibes the host blood components it ingests the antibodies, which attach themselves to the antigen. This results in its digestive processes being compromised, leading to starvation, loss of fecundity and weakness. The parasite will then detach and be carried out of the gut by peristalsis (Smith, 1999). A large amount of work has been involved in developing vaccines based on these hidden antigens, particularly molecules associated with the gastrointestinal tract. The potential importance of vaccinations with preparations containing proteins associated with the luminal surface of the parasite's gastrointestinal tract was initially shown by Munn *et al* (1987) against experimental haemonchosis in sheep. The investigation of other possible protective antigens associated with the microvillar membrane, have identified integral membrane glycoproteins, H11 (Munn *et al.*, 1993) and H-gal-GP (Smith *et al.*, 1994).

Immunisation with H11 and H-gal-GP has been shown to be 90% and 72% effective, respectively, in reducing challenge *H.contortus* worm burdens in sheep.

Table 1.2 adapted from Smith (1999) displays the gastro-intestinal nematode vaccine antigens that have given substantial protection in cattle, sheep and goats. Considerable advances have been made towards the development of commercial nematode vaccines for sheep in the past few years. Many protective antigens have been reported and the genes encoding some of these have been cloned. However, there is still a large amount of work to be carried out before a vaccine for nematodes is commercially viable. The main problem is the economical production of recombinant versions of the protective antigens (Newton, 1995; Knox & Smith, 2001).

						% Protection		
Parasite	Antigen (Ag)	Ag type	Mol. Wt (kDa)	Gene(s) Isolated	Identification /Function	Eggs	Worms	Host / Ref
H.contortus	H11	H	110	3	Aminopeptidases	94	90	Sheep / 1, 4, 7
	H-gal-GP	H	91,41	4	Metallopeptidases	93	72	Sheep / 5, 8, 9
	complex		42,40	1	Pepsin/pepsinogen			
	Thiol biding	H	40-55	3	Cysteine proteases	92	53	Sheep / 6, 12
	GA1	H	46,52	1		50	60	Goats / 10, 13
	H45 or P150	H	45,49,53			69	30	Sheep / 4, 14
	complex							
	ES15 +24	N	15 & 24			73	82	Sheep / 2, 3
T.circumcincta	ConA	H	Various			80	58	Sheep / 11
	Thiol	H	?			71	65	Sheep / 6
O.radiatum	Adut worm	?	60			?	93	Cattle / 15
	extract							

H = Hidden, N = Natural,? = Not known

1 Newton, 1995
2 Schallig *et al.*, 1997
3 Schallig *et al.*, 1997
4 Smith *et al.*, 1993
5 Smith *et al.*, 1994
6 Knox *et al.*, 1995
7 Smith *et al.*, 1997
8 Redmond *et al.*, 1997
9 Longbottom *et al.*, 1997
10 Jasmer & McGuire, 1991
11 Smith *et al.*, 1993
12 Redmond *et al.*, 1997
13 Jasmer *et al.*, 1996
14 Munn *et al*, 1997
15 East *et al*, 1993

Table 1.2 Candidate GI nematode vaccine antigens in sheep, goats and cattle

1.10 MARKERS FOR SELECTIVE BREEDING

Selection for parasite resistance in sheep has been undertaken in many countries to try and tackle the current problems of anthelmintic resistance (Beh & Maddox, 1996). The first stages in this selection procedure are to recognise markers for resistance to gain a more detailed understanding of the genetic mechanisms underlying resistance (Schwaiger *et al.,* 1995; Beh & Maddox, 1996; Woolaston & Baker, 1996; Paterson *et al*, 1996). It may not be feasible or even advantageous to identify animals that have genetic resistance to nematode infection, but it has been shown that some of the natural variations in resistance are under genetic control (Wakelin, 1978; Barger, 1989; Stear & Wakelin, 1998; Gray and Gill, 1993; Raadsma *et al.,* 1998; Stear & Wakelin, 1998). Selection for nematode resistance through traditional breeding programmes have hitherto been successful and flocks of sheep with high levels of resistance have been established (Windon & Dineen, 1980; Albers and Gray, 1987; Windon 1990; Woolaston *et al.,* 1990). The identification and characterisation of some of the genes associated with regulating resistance will mean that early selection of genetically responsive animals may increase the rate of selection for resistance (Albers and Gray, 1987). For all selection programmes to be successful, it is vital that the responsive individuals can be correctly and efficiently identified.

FECs have been demonstrated to possess a high correlation with worm burdens (Baker *et al.,* 1990; Stear *et al.,* 1995a; Bisset *et al.,* 1996) and it is therefore suggested that they could be thought of as a direct measure of nematode infection. In Australia and New Zealand, selection programmes involving resistant and susceptible lines of sheep using FECs as the selection criteria have been established for several years (Douch *et al.*, 1995). However, the faecal egg count method has several disadvantages. Faecal egg counting is labour intensive and time consuming, and samples cannot be stored for any length of time. Additionally, faecal egg counts may be influenced by external factors such as diet, quality of pasture and grazing habits. Also, for the less fecund species such as *Teladorsagia* there may be a poor relationship between worm burden and faecal egg output. For these reasons it is important to identify genetic and immunological markers

that accurately reflect the immune status of lambs and their ability to resist parasitic infection.

The use of genetic markers to define the genotype and foresee the performance of an animal is a significant support to animal breeding. DNA markers are regarded as polymorphic and the procedures undertaken to characterise these markers include restriction fragment length polymorphisms, single nucleotide polymorphisms and microsatellites (Beuzen *et al.,* 2000). Coltman *et al.,* 2000 demonstrated that a microsatellite polymorphism (nucleotide repeats interspersed throughout the genome) in the gamma interferon gene is associated with resistance to gastrointesinal nematodes in a naturally parasitised population (predominantly *T.circumcincta*) of Soay sheep. There are many future prospects for the use of genetic markers including DNA chip technology that could transform animal breeding (Beuzen *et al.,* 2000). Practical limitations to these approaches may involve high cost, the requirement of expertise and lack of literature although information on these strategies is growing exponentially.

Immunological factors generally reflect the host response to infection. Examples of these factors include parasite specific antibodies and peripheral eosinophil levels. Studies have demonstrated that peripheral eosinophil levels are associated with resistance in *T.colubriformis* infected lambs (Dawkins *et al.*, 1989; Buddle *et al.*, 1992). However, it was reported in several other studies that peripheral eosinophilia was inconsistent and variable for *H.contortus* and *T.colubriformis* resistant and susceptible lines of sheep, which questioned its use as an indicator (Douch *et al.,* 1996; Woolaston *et al.,* 1996). Studies have also demonstrated that lambs selectively bred for resistance had markedly higher levels of parasite specific IgG1 and IgM antibodies compared to the susceptible animals (Bisset *et al.,* 1996).

Immunological markers that are under current investigation by both the Moredun Research Institute, Edinburgh and at Glasgow University include the immunoglobulins E and A. Previous studies have demonstrated that small ruminants can regulate their worm populations by effector mechanisms, which can act upon parasite establishment, development, fecundity and persistence. Recent investigations at Glasgow University

with Scottish Blackface lambs have reported that a decrease in female worm length was associated with an increase in local IgA response to fourth stage larvae. Additionally, the number of inhibited larvae was positively associated with the size of the IgA response and positively associated with the size of the worm burden (Stear *et al.*, 1995b). This suggests that IgA may play a key role in mechanisms regulating parasite development and fecundity (Stear *et al.*, 1995b; Strain *et al.*, 2002).

The integration of immunological markers for selective breeding may be an additional way of controlling the nematode population in sheep. Individual animals within a flock are more, or less resistant to nematode parasites and the reasons behind this probably relate to immune function. It is therefore important to understand all the aspects of immunity involved in the response to nematode infection because if immune markers of resistance can be identified then they would fulfill a crucial role in selective breeding programmes.

1.11 IMMUNE RESPONSE TO GASTROINTESTINAL NEMATODES

Firstly, it is important to consider that different patterns of nematode expulsion are recognized. Three patterns of worm expulsion have been observed in the immune response to gastrointestinal nematodes in both laboratory animals and ruminants and are outlined below.

In the first example, ingested larvae are excluded from their niche and are thereafter lost rapidly from the immune animal. This process termed 'Rapid Expulsion' (RE) occurs even before establishment takes place. Love *et al.*, (1976) in describing one of the early examples of RE, demonstrated a reduction in the burden of *Trichinella spiralis* in immune rats that were challenged 12 hours earlier. These results have been backed up in mice (Wakelin & Lloyd, 1976). Another example of RE has been reported by Miller *et al* (1983) and Jackson *et al* (1988) describing the RE of exsheathed *Haemonchus contortus* larvae (within 48 hours) from hyperimmune sheep. Suggested mechanisms for the immune expulsion of incoming larvae have been suggested. These include roles

for complement, the cooperation between mucus and antibodies and their interaction with inflammatory mediators released from mast cells and granulocytes (Miller, 1984).

In the second pattern of expulsion the infective larvae reach their niche and develop before they are expelled. Expulsion appears to be associated with a particular stage of their life cycle, for example moulting (Soulsby, 1985). This event termed the 'self-cure phenomenon' suggests that the sudden ingestion of a high dose of infective larvae coincides with the expulsion of an existing population of established worms. However, the worm expulsion does not seem to occur until about the period of larval exsheathment (Stewart, 1953) suggesting that this occurrence is characteristic of a stage-specific process associated with an immediate hypersensitivity response (Stewart, 1955). It is considered that this 'self-cure' is likely to be advantageous to both host and parasite. The host would gain a momentary respite from sustainable blood loss while the older parasite population is eventually replaced by an active younger generation (Urquhart, 1991).

In the third pattern, expulsion of adult worms occurs over the days, weeks and months post-infection. Jarrett & Urquhart (1969) described that in primary infections of *Nippostrongylus brasiliensis* in rats, the establishment and expulsion of adult worms follows a characteristic pattern. This pattern begins with the loss phase 1 – a number of incoming larvae are lost; plateau phase – establishment and maturation of the survivors; loss phase 2 – expulsion of adults; and threshold phase – small remaining population (Rothwell, 1989). A similar pattern has been observed following infection of helminth naïve hosts with other nematodes, for example; *T.spiralis* in rats (Rothwell, 1989), *Trichuris muris* in mice (Wakelin, 1967), *T.colubriformis* in guinea pigs (Rothwell & Griffiths, 1977), and *H.contortus, T.circumcincta* and *O.ostertagi* (Michel, 1976) and *N.battus* (Lumley & Lee, 1981) in ruminants. The kinetics of this characteristic feature of worm establishment and expulsion depends on the host-parasite combination, the host immune status, host genotype and the level of infection.

1.12 IMMUNOLOGICAL DEFENSE MECHANISMS TO GASTROINTESTINAL NEMATODES

The outcomes involved in the immune response to gastrointestinal nematodes have been outlined above. Aspects of specific mechanisms have been suggested and are discussed below.

1.12.1 Humoral (Antibody) response

A number of early studies on the development of immunity have observed increased levels of parasite-specific antibodies in gastrointestinal nematode infections of sheep. However, there is still some uncertainty over the role of these antibodies in the expulsion of gut parasites (Miller, 1984).

The immune system of sheep consists of IgG_1, IgG_2, IgM, IgA and IgE isotype antibodies. Studies have primarily focused on both systemic and locally produced IgA and IgG (Miller, 1996). The role of IgM is often thought to be of less importance (Schallig *et al*, 1995). Evidence for the significant role of IgE antibodies in the immune response against gastrointestinal nematodes is rapidly emerging.

A number of different mechanisms for antibody activity have been suggested. These consist of facilitating the attachment of phagocytic cells, activating complement, interfering with nutrition by blocking vital parasite enzymes (Smith *et al.*, 1985; Gill *et al.*, 1994), inhibiting fundamental metabolic processes vital for establishment and maintenance (Carlisle *et al.*, 1990), and degranulating mast cells, basophils and eosinophils (Huntley, 1992).

In monogastric animals, IgA is the main antibody isotype locally secreted in the gastrointestinal tract and is important in the protection against gut bacteria, bacterial toxins and viruses (Jeffcoate *et al.*, 1992). Elevated concentrations of parasite specific antibody predominantly the IgA isotype have been demonstrated in the abomasa of *H.contortus* infected sheep. After challenge, IgA in the gastric lymph of immune sheep increases significantly (Miller, 1984). Studies have also shown that serum IgA antibody titres were higher in resistant sheep compared with randomly bred sheep after challenge

infection (Gray *et al.*, 1993). Investigations have also reported a significant correlation between the mucosal IgA antibody response and resistance to challenge infection in calves that were previously infected with *Ostertagia ostertagi* (Claerebout *et al*, 1999). The exact role of IgA in the immune response against gastrointestinal nematodes in ruminants is however unclear. Stear *et al.* (1995b) suggested that the length of the worm and fecundity were regulated by the amount and specificity of the local IgA response. It has also been shown that parasite specific IgA antibodies in the abomasal mucus were negatively correlated with FECs and the number of eggs per female worm, suggesting that local IgA responses in the abomasum are associated with a reduction in worm fecundity (Claerebout, 1999).

Increased concentrations of serum IgG and IgM levels are evident in sheep in the response to nematode infection. Local IgG antibodies have also been observed in *T.circumcincta* infected sheep (Smith *et al*, 1983). Higher levels of IgG1 were observed in the serum of sheep bred for resistance to *H.contortus* after challenge infection compared to randomly bred sheep. However, no differences were found in IgG2 and IgM responses. A negative correlation was also determined between FEC and serum IgG1 but not with IgM or IgG2 (Gill *et al*, 1993).

An increase in total and parasite-specific IgE antibody is generally viewed as an important factor in the host response to helminth infections (Jarrett & Miller, 1982; Hagan, 1993; Pritchard, 1993). Most of the investigations on IgE responses to helminth infections have been in humans and rodents. Until recently, the role of IgE in the protection against helminths in ruminants has been studied less extensively. However, specific antibodies against bovine (Thatcher & Gershwin, 1988) and ovine (Shaw *et al*, 1996; Kooyman *et al*, 1997) IgE have now become available. It is well documented that helminth infections in humans, rodents and ruminants are associated with an increase in total and parasite specific IgE levels (Hagan, 1993; Huntley *et al.*, 1998a). The number of mast cells, eosinophils and basophils also increase during gastrointestinal nematode infections in sheep providing an important role for IgE as it binds, via the high affinity (FcεRI) receptor to all of these cells, and on being cross linked by antigen, stimulates

degranulation releasing molecules which possess anti-parasite activity. IgE can also bind to low affinity receptors (FcεRII/CD23) in sheep, which are found on a number of inflammatory cell types. Harrison *et al.* (1999) have suggested additional evidence for the function of IgE in the expulsion of challenge infections through its involvement in immediate hypersensitivity reactions. It has been demonstrated in many studies that an IgE response to different antigens at different stages of development is produced. During a primary response, IgE antibody levels to antigens obtained from L_3 and adult *T.circumcincta* were negligible with limited concentrations of IgE antibody detected in the gastric lymph and serum samples. However, a noticeable IgE antibody response to L_3 antigens in over half of the sheep was demonstrated 2-8 days post-challenge of primary infected sheep. In contrast, low levels of IgE antibody to adult antigens were observed (Huntley *et al*, 1998b). By further contrast however, Kooyman *et al* (1997), demonstrated an evident IgE response against adult ES antigens of *H.contortus*. Additionally, a systemic IgE response in sheep to primary and challenge *T.colubriformis* infections and peak IgE responses to adult antigens between 20-27 days after infection plus secondary IgE response to both adult and L_3 ES antigens have been demonstrated (Shaw *et al*, 1998).

1.12.2 Cell-mediated immunity

The immune response in sheep to gastrointestinal nematodes is thought to be facilitated by CD4+ T cells produced in the mesenteric lymph nodes (Gill *et al*, 1992). CD4+ T cells are vital for worm expulsion, as demonstrated by investigations in athymic mice (Wakelin, 1978). A number of experimental models, the majority being murine, have shown that CD4+ T cells can be divided into two subsets of T-helper cells designated T helper type 1 (Th1) and T helper type 2 (Th2). This subset classification is based on the cytokines that they secrete (Mosmann *et al*, 1986; Mosmann & Coffmann, 1989). Th1 cells generate a number of cytokines including interleukin (IL) 2, interferon gamma (IFN-γ) and tumour necrosis factor beta (TNF-β) resulting in a cell mediated response. Th2 cells produce IL 4, 5, 6, 9, 10 and 13 (Else *et al.*, 1992; Bancroft &

Grencis, 1998). Th2 is the main T cell population triggered after helminth infection (Grencis, 1997). A typical Th2 response is distinguished by increased immunoglobulin secretion by B-cells, in particular IgE and IgG_1, and the production of eosinophils and mast cells. Prior studies have demonstrated an association between Th1 responses and susceptibility to gastrointestinal nematode infections (Bancroft & Grencis, 1998). This evidence, therefore suggests that the production of different Th subsets may contribute to variation in host resistance. Th cells have been shown to facilitate worm expulsion from laboratory animals and to control mastocytosis, eosinophilia, goblet cell hyperplasia and other characteristic gut pathology (Bancroft & Grencis, 1998; Grencis, 1996; Laurence *et al.*, 1998). In sheep, lymphocytes have also been demonstrated to be significant in immunity against gastrointestinal nematodes. Transfer of gastric lymph lymphocytes from lambs, immunised against *H.contortus* (Smith *et al.*, 1984a) or *T.circumcincta* (Smith *et al.*, 1986) to their genetically identical uninfected twins, transferred protection against a homologous challenge infection (Smith *et al.*, 1984a, 1986). Moreover, the vital importance of T helper cells in the protective immune response against *H.contortus* has been demonstrated with an *in vivo* decline of CD4+ T cells abrogating immunity to *H.contortus* in genetically resistant sheep (Gill *et al.*, 1993b). Other recent studies have shown that the *in vivo* decline of CD4+ T cells partly abolishes immunity to *H.contortus* stimulated by gut membrane immunisation (Kuranu *et al.*, 1997). It still remains unclear whether there are two distinct subsets of T helper cells in ruminants. However, there are various characteristics of nematode infection in ruminants, which would be considered 'Th2-like' if they had occurred in the mouse (Claerebout & Vercruysse, 2000).

In association with the major aspects of the immune response outlined above, it is appropriate to afford specific comment in relation to the many cells believed to play an effector role in nematode expulsion.

1.12.3 Mast cells

One of the most noticeable features of gastrointestinal nematode infections in rodents and ruminants is the production of mucosal mast cells (MMC) (Miller, 1996). Mast cells

as mentioned previously, have high affinity receptors (FcεRI) for IgE antibodies. Degranulation occurs when the membrane bound IgE is cross-linked by antigen releasing mediators such as histamine, chymase (mast cell protease), tryptase, serine esterases and tumour necrosis factor alpha (TNF-α). Additionally, there is evidence that ovine mast cells synthesise and secrete leukotrienes and other molecules, which have nematode larval migration inhibitory activity (Douch *et al*, 1995). Activation can also be triggered by IL 1 and 3, granulocyte-macrophage colony stimulating factor and certain complement factors, C3a and C5a. Two sub populations of ovine mast cells have been observed: mucosal mast cells and connective tissue mast cells (Huntley, 1992). Gastrointestinal nematode infections in ruminants are generally associated with mucosal mast cell hyperplasia (Miller, 1996). Several studies have shown that mast cells are activated during the expulsion of nematodes in sheep (Huntley *et al.*, 1987; Emery *et al.*, 1993). However, it has also been shown that sheep can express a high level of resistance with no noticeable increase in mast cell numbers (Huntley, 1992). The suppression of mucosal mast cells in sheep by treating the animals with corticosteroids abrogated the rapid expulsion of nematodes (Huntley, 1992). Huntley (1992) also demonstrated that the concentrations of sheep mast cell protease, an enzyme located in the MMCs and globule leucocytes (Huntley *et al*, 1986), increase in the serum and mucus of immune sheep but not susceptible sheep after direct abomasal challenge with L_3 *H.contortus*. Stear *et al* (1995b) demonstrated an association between the *T.circumcincta* worm burden of six-month-old sheep and the number of globule leukocytes (degranulated mast cells). It is suggested that mast cells contribute to the expulsion of worms possibly by means of an immediate hypersensitivity reaction (Stear *et al.*, 1999). Studies in mast cell deficient mice (w/w) have shown that the expulsion of *N.brasiliensis* can occur without mastocytosis (Crowle, 1983), whereas the expulsion of *T.spiralis* is mast cell dependent (Woodbury *et al.*, 1984). This raises the possibility more than one mechanism may be operating in a `hierarchal` fashion, such that if one mechanism is blocked there is an efficient back-up system (Rothwell, 1989). Consequently, there is probably not one overall mechanism of expulsion (Maizels & Holland, 1998).

1.12.4 Granulocytes

Circulating and tissue eosinophilia is a regular characteristic of helminthiasis. Eosinophils have been demonstrated to be associated *in vivo* with the rejection of several helminths through treatment of mice or guinea pigs with anti-eosinophil serum or anti-IL5 monoclonal antibodies during infections (Rainbird *et al.*, 1998). Eosinophils establish themselves in the tissue of invasive L_3 of sheep gastrointestinal parasites (Rainbird *et al*, 1998). Associations between elevated eosinophil levels and the ability of lambs to respond to *T.colubriformis* and *H.contortus* infections have been demonstrated (Barrett, 1997). In contrast, negligible differences have been observed in the number of circulating or tissue eosinophils in non-infected, primary or secondary infected sheep in investigations by Huntley *et al* (1995) and Schallig *et al* (1997). These results all suggest that eosinophils do not seem to have a direct effector role against gastrointestinal parasites. An indirect role, however, cannot be disregarded. Basophils are also thought of as another possible source of mediators. Circulating basophils and infiltration of the site of infection by these granular cells is associated with mast cell proliferation and the expulsion of *T.colubriformis* from guinea pigs (Rothwell, 1989).

1.12.5 Mediators

In this context, mediators are defined as substances with potent biological effects released from a number of different cells such as mast cells, basophils, eosinophils, macrophages, platelets and T cells. Various antibody isotypes, complement, cytokines, eosinophil products, platelet substances and nematode antigens may serve to induce mediator release. However, cross-linkage of mast cell membrane bound IgE by specific antigen is, perhaps, the predominant mechanism involved. There are many types of mediators including leukotrienes, prostaglandins, interleukins and other cytokines (Rothwell, 1989). Rothwell (1989) discusses three hypotheses about the function of mediators in the expulsion process in rodents. Firstly, the hypothesis known as the 'leak lesion' suggests that in primary *N. brasiliensis* infections in rats, mediators that are produced from a mucosal hypersensitivity reaction increase the permeability of the

mucosa allowing specific anti-worm antibodies to leak through the lesion and attach to the nematode causing their expulsion from the host. Secondly, mediators may be involved in mucosal inflammation at the site of infection causing the microenvironment of the worms to deteriorate leading to expulsion from the host (Rothwell, 1989). The final hypothesis proposes that mediators may have a direct effect on worms. For example, they may affect the function of sensory structures, disorientate the worms and prevent establishment in their niche (Ogilivie & Jones, 1973).

In sheep, the accumulation of globule leukocytes in the gut mucosa has been associated with the rejection of the intestinal dwelling nematodes, *T.colubrifomis* (Dineen *et al.*, 1978; Douch *et al*, 1986) and *N.battus* (Winter *et al.*, 1997), and the abomasal dwelling nematodes *H.contortus* and *T.circumcincta* (Smith *et al.*, 1984b; Stear *et al.*, 1995b). Douch *et al.* (1983) studied the antiparasitic property of mucus from immune sheep using an *in vivo* larval migration inhibition assay. Inhibition of *T.colubriformis* larval migration was attributed to mucus components sharing similar properties to slow reacting substance of anaphylaxis (SRS-A). Mucus also inhibited the migration of abomasal species larvae. The major constituents of SRS-A are the leukotrienes B4, C4, D4 and E4, two of which were assessed alongside histamine, in lambs genetically defined as high or low responders to vaccination and challenge with *T.colubriformis* (Jones *et al.*, 1990). Elevated concentrations of these mediators were observed in the mucus of responsive animals (relative to intestinal tissue) and it was suggested that they might play a role in preventing the establishment of incoming larvae (Jones *et al.*, 1990). Jones *et al.* (1994) also used a larval migration inhibition assay to examine the potential causal relationships between mediator release and kinetics of parasite rejection. Challenge of immune sheep induced the release of mediators and the intestinal contents inhibited migration of larvae significantly more than those of worm-free sheep given a single infection of *T.colubriformis*. Mediator concentrations in gut contents were noticeably lower than those found in mucus indicating that the adjacent mucosa was the probable source of these mediators. Increased concentrations of mucosal mediators were apparent at the time when egg counts decreased in primary

infected animals suggesting a function for these mediators and/or their cellular source in the expression of immunity (Jones *et al.*, 1994).

1.12.6 Mucus & Goblet Cells

Structural and functional alterations to mucosal epithelia, enteric goblet cells and gastric mucous cells have been described at length in parasitised mucosae (Miller, 1987; Nawa *et al*, 1994). These changes are associated with altered motility, smooth muscle hyperplasia, and increased fluidity in the gut lumen (Emery *et al.*, 1993). Studies suggest that many of these changes also appear in parasitised sheep (Emery *et al.*, 1993; McClure *et al.*, 1992). It therefore seems that mucus and motility may be common and key components of the mammalian expulsion.

Hyperplasia of host gastrointestinal tract mucus secreting goblet cells has been described in a range of nematode infections in rodents and sheep (Miller, 1987) and there are elevated levels of mucous cells in parasitised ovine gastric mucosa (Newlands, Miller & Jackson, 1990). It has been documented that in *N. brasiliensis* infections in rats, there is a temporal link between this hyperplastic response and the expulsion of a primary infection (Rothwell, 1989). Additionally, drugs interfering with mucus function inhibit worm expulsion (Miller & Huntley, 1982). The findings in *N.brasiliensis* and other nematodes have led to the idea that mucus has a protective function. Together with antibody and complement, mucus may dislodge established worms or may envelop and trap incoming worms therefore excluding them from their niche and hence favouring their expulsion (Miller, 1984).

1.13 AIM

The main aim of my project is to identify immunological markers associated with resistance and susceptibility, and particularly to define the relative importance of IgE and IgA in the immune response of sheep to gastrointestinal nematodes. Recent studies at Moredun Research Institute on the mechanisms of immunity to nematodes in sheep have demonstrated that the IgE response is directed predominantly to the L_3 stage of

Teladorsagia circumcincta larvae. This has led to the identification of a specific *Teladorsagia circumcincta* L_3 antigen that plays a major role in the stimulation of local IgE antibody production (Huntley *et al.*, 2001). Initial studies will further characterise this allergen of interest in order to define its physicochemical properties. The high molecular weight L_3 antigen will be used in ELISA assays to determine the concentrations of various parasite specific antibodies (IgE, IgA and IgG) present within lambs naturally infected with *T.circumcinta*. Other immunological markers to be measured will include peripheral eosinophils and IgE bearing cells. The outcome of all of these investigations will potentially provide more information in relation to defining the underlying mechanisms of immunity against gastrointestinal nematodes in sheep. Immunological markers associated with responsiveness against nematodes have the potential to be used as supplementary phenotypic markers for individual responsiveness and herd responsiveness. A phenotypic marker for individual responsiveness would be useful for the selection of responder line animals in breeding programmes and for herd responsiveness a marker would be useful for strategic chemoprophylaxis.

Chapter 2

General Materials and Methods

2.1 ANIMALS

2.1.1 Greyface Suffolk Cross lambs

Greyface Suffolk Cross parasite naïve lambs used in this study were born and reared indoors for the first three weeks under conditions designed to prevent accidental infection. They were then put out onto pasture on the paddocks at Moredun Research Institute's Firth Mains farm. Previous results have shown that in this environment the strongyle populations are dominated by *T.circumcincta* (Barrett, 1997).

2.1.2 Scottish Blackface lambs

Scottish Blackface parasite naïve lambs used in this study were born and reared indoors for the first three weeks under conditions designed to prevent accidental infection. They were then put out on to pasture on the paddocks at Moredun Research Institute's Firth Mains farm, which were predominantly infected with *T. circumcincta* (Barrett, 1997).

2.2 PARASITOLOGICAL TECHNIQUES

2.2.1 Collection of faecal samples

Rectal faecal samples, weighing between 1 and 10 grams were collected from sheep into a polythene sample bag (300X 200mm, 1000 gauge) (Mac Kinnon & Hayes). The samples were processed in the laboratory and were examined within three days of storage at 4°C.

2.2.2 Scoring faecal samples

The faecal consistency of the rectal samples was monitored while the samples were being weighed for the faecal egg count method. A scoring system was allocated for the faeces, which is described in table 2.1.

Score	Consistency of faeces
1	Blood and Mucus
2	Fluid sample of faeces containing blood and/or mucus
2-3	Fluid sample of faeces, no blood or mucus
3	Soft unformed faecal material
3-4	Soft, partially formed pelleted faecal material
4	Moist pelleted faecal material
5	Dry, hard pelleted faecal material

Table 2.1 Scoring system used to monitor faecal consistency

2.2.3 Faecal egg count method

The gastrointestinal nematode egg population of a faecal sample was determined using the flotation technique as described by Jackson (1974). The rectal faecal samples were weighed and 10 ml of tap water was added per gram of faeces. A stomacher (Stomacher 80, Lab-Blender, London, UK) was then used to emulsify the faecal samples. A 10 ml sub-sample was then removed and passed over a 1mm sieve (John Lewis, UK) and washed through with an additional 5 ml of tap water. The retentate was squeezed to remove as much fluid as possible. The filtrate was poured into 15 ml polyallomer centrifuge tubes (16 X 102mm, Beckman, USA) and centrifuged at 1000rpm for 2 minutes.

The supernate was removed using a vacuum line, leaving a 1 ml pellet containing faecal debris and eggs. The pellet was re-suspended with 10 ml saturated sodium chloride (NaCl) solution and then the tubes were centrifuged again at 1000rpm for 2 minutes. Using artery forceps, the tubes were clamped just below the meniscus of the supernate and the contents of the upper chamber were poured into a 4 ml disposable polystyrene cuvette (LIP Ltd, Shipley, UK). The upper chamber of the tube was rinsed with a further 1 ml of saturated NaCl solution and the solution added to the cuvette. The cuvette was inverted to re-suspend the eggs uniformly, filled with NaCl solution and then sealed with a cuvette lid. The cuvette containing all of the eggs recovered from one gram of faeces was counted using a compound microscope (Leica DMLS) at the lowest magnification (X40). The microscope contained a calibrated eyepiece graticule (Miller square, Graticules Ltd, UK), which was used to

count the eggs in the faecal samples that had high numbers of eggs present. All the eggs were counted for samples that only contained a few eggs (<50). If samples consisted of high levels of eggs (>50), the graticule was used. If eggs fell in the large square along two traverses of the cuvette the eggs were multiplied by three and if the eggs fell in the small square they were multiplied by nine in order to obtain the total number of eggs per gram (epg) of faeces.

2.2.4 Nematode Parasite Isolates in culture at Moredun Research Institute

The Moredun ovine susceptible isolate of *T.circumcincta* was isolated from the fields at Moredun Research Institute in 1991. Since isolation it has undergone a total of 31 passages through parasite naïve lambs.

The ovine susceptible isolate of *T.colubriformis* was obtained from the Central Veterinary Laboratory at Weybridge in 1998. Since then it has undergone 7 passages in parasite naïve lambs.

The ovine susceptible isolate of *H.contortus* was originally obtained in the mid 1950's from Glasgow University and is believed to have been isolated at the National Veterinary Research Centre in M'guga in Kenya. Since then it has been passaged on average about three times per annum using parasite naïve lambs.

The Moredun ovine susceptible isolate of *C.curticei* was isolated from the fields at Moredun Research institute's Firth Mains Farm in 1976, since isolation it has undergone passage twice using parasite naive lambs.

The Moredun ovine susceptible isolate of *N.battus* was isolated from the fields at Moredun Research Institute's Firth Mains farm in 1998, since isolation it has undergone passage twice using parasite naïve lambs.

2.2.5 Recovery of infective larvae from faeces

Faeces were collected from donor animals infected monospecifically with a known parasite. The collected faeces were placed into a plastic culture tray (400 X 200 X 75mm) to a maximum depth of approximately 3cm and the tray was loosely sealed inside a polythene bag (500 X 300mm, Mac Kinnon & Hayes). The bag was punctured to allow airflow. The tray was incubated at 22°C for ten days. The tray

was then flooded with tepid tap water (22°C) and the faeces were allowed to soak for between two and four hours. The contents of the tray were sieved through a 1.0mm sieve and the filtrate was collected. The filtrate was sedimented for 2 hours at 4°C and then the volume was reduced by using a vacuum line. The larvae were extracted from the remaining filtrate using the Baermann apparatus (see figure 2.1).

Two layers of high wet strength paper were placed over a filter holder and attached with an elastic band. A 250 ml beaker or jam jar was filled with tepid water (approximately 22°C). The filtrate prepared previously was poured onto the high wet strength paper of the Baermann apparatus, ensuring that the sample was distributed evenly over the paper. The Baermann apparatus was then immersed in the warm tap water in the beaker and incubated at room temperature for two hours. In this time, the infective larvae migrated through the Baermann apparatus into the beaker of tap water.

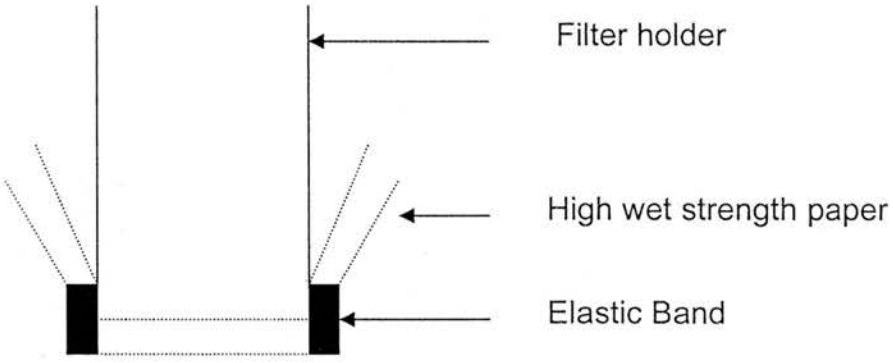


Figure 2.1 Baermann apparatus

2.2.6 Determination of the number of infective larvae

The larval culture was added to a 1000ml volumetric flask and the volume was made up to 1000ml with tap water to provide a stock suspension. Inverting the flask several times ensured an even suspension. 1ml stock suspension was then added to 9ml tap water in a 10ml volumetric flask to make a 1/10 dilution. If large numbers of larvae were present in the stock suspension then the above step was repeated to give

a 1/100 dilution. The volumetric flask was then inverted several times and a 100µl sub-sample of the diluted suspension was streaked along a labelled non-frosted microscope slide. The numbers of live larvae were determined using a compound microscope (Leica DMLS) with a mechanical stage at X40 magnification. The last two steps were repeated a further 4 times. The average number of larvae per slide was calculated and then multiplied up to calculate the numbers of larvae per ml of the stock suspension. This was achieved by multiplying by 10 to calculate the average number of larvae per ml of the diluted sample. Then by dilution (X10 for single dilution, X100 for two dilutions). The total number of larvae present was then calculated (X1000). The volume was adjusted to the required concentration if necessary. The infective larvae were stored in a Duran Bottle in the refrigerator at + 4°C for several months.

2.2.7 Recovery of L_4 and L_5 stage *T.circumcincta* larvae from infected animals

Sheep were killed 6-8 days post infection for the collection of L_4 larvae and 10-28 days post infection for L_5 larvae. Larvae were harvested from the abomasum and immediately placed into 30ml of PBS in a 50ml conical centrifuge tube (Corning, USA) prior to being frozen and maintained at -70°C.

2.2.8 Preparation of soluble extract from different stages of larvae

Approximately 3 X 10^6 of infective L_3 larvae were used and these were taken from a stock that had been stored at 4°C in tap water for up to one month. The L_3 larvae were transferred to a 50ml conical centrifuge tube and allowed to settle under gravity. The L_4 and L_5 larvae collected in 2.2.7 were thawed and used in the same following procedure as the infective L_3 larvae.

Excess water (or PBS for L_4 and L_5 method) was aspirated from the surface of the centrifuge tubes. The volume of packed larvae was noted and an equal volume of high density Zirconium silicate beads (cleaned, 1mm diameter) (Fox Industries Inc., USA) was added to the tube and gently mixed. The mixture was then transferred to a mortar and thoroughly homogenised using a pestle. This procedure was carried out on ice. The homogenate (including beads which remained intact) was than aliquoted

into 1.5ml eppendorfs (Elkay, Co.Galway) and each eppendorf was centrifuged at 9000rpm for 5 minutes (Mini Spin Plus, Eppendorf, Germany). The supernatant, containing soluble protein, was then aspirated and pooled. The remaining beads were rinsed five times with distilled water for re-use. A Pierce BCA protein assay (PIERCE, Rockford, USA) (see 2.5.4) was performed on the supernatant according to the manufacturer's instructions, to determine the protein concentration. Soluble protein, which was termed "whole larval antigen" was then realiquoted into 1ml volumes and stored at –20°C. These antigens were employed in a variety of immunological techniques described later.

2.3 PRODUCTION PARAMETERS

2.3.1 Weighing

Animals were weighed to the nearest half-kilogram using a calibrated weigh crate, at a similar time of day on each visit.

2.3.2 Collection of blood

Blood was taken from animals by jugular venepuncture into 7ml vacutainer tubes (Becton Dickinson, UK) containing ethylenediaminetetraacetate (EDTA). Animals were bled at a similar time of day on each visit.

2.3.3 Plasma Samples

Blood samples collected in *2.3.2* were centrifuged at 3000 rpm. (2060g) for 20 minutes. The plasma was carefully removed using a Pasteur pipette into two 2ml aliquots, which were frozen at –20°C for subsequent analysis.

2.4 IMMUNOLOGICAL TECHNIQUES

2.4.1 Ammonium sulphate precipitation of colostrum for use as a positive control in ELISAs and Western Blots

Prior testing determined that colostrum from infected sheep contained high levels of IgE, IgA and IgG antibodies to *T.circumcincta* L_3 antigen (J.Pettit, unpublished observations) and for uniformity, one sample was selected to provide the positive control throughout these studies. The selected colostrum sample was obtained from

sheep number 1832X from Experiment GY carried out at the Moredun Research Institute in 1998 and stored at –20°C. The colostrum sample was diluted 1 in 4 in PBS to give a total volume of 100µl. The sample was then heat inactivated for 30 minutes at 56°C. 400g of ammonium sulphate was dissolved in 500ml of distilled water to prepare a saturated solution of ammonium sulphate and 43µl of this saturated ammonium sulphate was added to the 100µl of heat-inactivated colostrum dilution and this was immediately vortexed. This solution was then centrifuged for 5 minutes at 10,000rpm in a microfuge (Mini Spin Plus, Eppendorf, Germany). The supernatant was carefully pipetted into a clean labelled eppendorf and the pellet was discarded. 57µl of saturated ammonium sulphate was then added to the supernatant and the eppendorf was vortexed immediately. The eppendorf was then incubated at +4°C overnight. Samples can be stored at this stage for several weeks at +4°C. The sample was then centrifuged for 5 minutes at 10,000rpm in a microfuge. The supernatant was discarded. The pellet was then resuspended in 100µl of PBS/Tween 80 + NaCl (Refer to appendix for preparation of this reagent). This colostrum sample was then used in either ELISAs or western blots as a positive control rich in IgE, IgA and IgG antibodies.

2.4.2 Enzyme Linked Immunosorbent Assays (ELISAs)

(Refer to appendix for preparation of reagents and buffers)

The general ELISA method undertaken throughout this thesis is outlined below. The identification and concentrations of the primary and secondary antibodies employed in the ELISA method are specifically described in the materials and methods of the following chapters.

ELISA plates (M129B, Dynatech Lab Ltd.) were incubated overnight at 4°C with antigen at a known concentration (50µl/well) in 0.1M carbonate coating buffer, pH 9.6. The antigen was then discarded and the plates were washed twice in Phosphate Buffered Saline, 0.05%Tween 20 (PBS T20). The wells were then blocked with PBS/Tween 80 NaCl (200µl/well) and incubated for 30 minutes at room temperature. The plates were then washed twice in PBS T20. The wells were then incubated with prepared serum samples in duplicate (primary antibody), diluted at a known concentration in blocking buffer (50µl/well) for 1 hour at room temperature. The

first column was used as a blank and incubated with blocking buffer only (50µl/well) and two of the wells (G12 & H12) were incubated with the previously selected positive control colostrum at 1 in 50 dilution with blocking buffer. The same conditions were applied to ELISA determinations involving serum samples. The samples were discarded and the plates were washed 6 times in PBS T20. The wells were then incubated with a secondary antibody, at a known concentration diluted in blocking buffer (50µl/well) for 1 hour at room temperature. The supernate was discarded and the plates were washed 6 times in PBS T20. The wells were then incubated with a goat anti-mouse biotinylated tertiary antibody (DAKO Ltd., Denmark, E0433), at a 1 in 2000 concentration diluted in blocking buffer (50µl/well) for 1 hour at room temperature. The supernate was discarded and the plates were washed 6 times in PBS T20. The wells were then incubated with a Streptavidin-Horse Radish Peroxidase (HRP) (DAKO Ltd., Denmark, PO397) conjugate at a 1 in 5000 concentration diluted in blocking buffer (50µl/well) for 30 minutes at room temperature. The supernate was discarded and the plates were washed 6 times in PBS T20. Finally, the wells were incubated with o-phenylenediamine dihydrochloride (OPD peroxidase substrate, SIGMA FAST tablet set, SIGMA, UK)(50µl/well) until colour had developed enough to give a reasonable Optical Density (OD) at 492nm (~15 minutes). The reaction was stopped by the addition of 2.5 M sulphuric acid (H_2SO_4)(25µl/well). The plate was read at 492nm using a plate reader (Dynex Technologies, UK). The same ELISA template was employed in all assays and an example of this is shown in table 2.2 below: -

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37	S41
B	L	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37	S41
C	A	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38	S42
D	N	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38	S42
E	K	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39	S43
F	↓	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39	S43
G	↓	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40	Positive
H		S4	S8	S12	S16	S20	S24	S28	S32	S36	S40	Control

Table 2.2 ELISA template

The following equation was used on all the samples for each ELISA plate tested to allow for plate to plate variation: -

$$\frac{1}{\text{Mean OD Positive control}} \times \text{Mean OD Sample}$$

2.4.3 Eosinophil counts

Carpentiers stain (450µl) (refer to appendix for preparation of reagent) was pipetted into labelled 1.5ml eppendorf tubes. 50µl of whole blood was added to the eppendorfs and the tubes were capped and vortexed. Samples were stored for a maximum of one month at room temperature. Duplicate samples (2 x 10µl) were pipetted into fast-read chambers (Immune Systems, Paignton, UK). Eosinophils were counted within the grid at X100 magnification on a compound microscope (Leitz, Laborluks), excluding those that fell into the grooves between the squares. The total number of eosinophils were divided by 100 to give the number of eosinophils x 10^9 per litre.

2.5 PROTEIN ANALYSIS

2.5.1 Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

(Refer to Appendix for preparation of reagents and buffers)

Discontinuous SDS-PAGE was conducted with 0.75mm thick 7.5% slab gels (MINI PROTEAN II; Bio-Rad, UK), using the system described by Laemmli (1979). The glass plates and spacers supplied with the electrophoresis apparatus were prepared and placed into a casting stand cassette supplied with the apparatus for pouring the gel. The gel was then prepared. A 7.5% resolving gel solution was prepared by using 2.5ml resolving buffer, 2.5ml acrylamide (Severn Biotech Ltd.,UK), 4.4ml distilled water, 0.5ml 20% sodium dodecylsulphate (SDS) and gently mixing the solution by swirling. The resolving gel was then polymerised by adding 10µl N,N,N',N'-tetramethylethylenediamine (TEMED, SIGMA, Germany) and 100µl 10% ammonium persulphate (APS) and poured immediately into the

cassette until the level of the gel was approximately 1cm from the top of the plates. 1ml 74% ethanol was added to level the meniscus. The gel was left to polymerise.

A 4% stacking gel solution was then prepared by gently mixing 1.4ml stacking gel buffer, 0.72ml acrylamide, 3.09ml distilled water and 0.14ml 20% SDS. The stacking gel was then polymerised by adding 10µl TEMED and 100µl 10% APS. Once the resolving gel was set the ethanol was poured off and the stacking gel solution was carefully layered on top of the resolving gel. A Perspex comb was immediately added for moulding the sample wells, avoiding air bubbles. The gel was allowed to polymerise.

The comb was then taken out and the gel sandwich was attached to the cell electrode assembly and was placed in an electrode tank. Running buffer was added to the top and bottom tanks, filling the top tank completely. The next step involved preparing the samples.

Samples were either treated with non-reducing sample buffer or reducing sample buffer and boiled for 3 minutes before they were loaded onto the gel at a known concentration (see individual chapters). Equal volumes of both the sample and the SDS buffers were added. For each gel, standard markers were used and these are described in more detail in the following chapters.

Once samples were added to the wells of the gel a constant voltage of 200 volts was applied across the gel until the bromophenol dye front was near to the base of the plates. The power was then switched off and the gel was removed.

2.5.2 Protein detection in SDS-PAGE gel using Colloidal Coomassie Stain

Proteins in SDS gels were detected using Colloidal Coomassie stain (Invitrogen, Carlsbad, CA). Following electrophoresis, the SDS-PAGE gel was carefully taken off the cassette and fixed in a 20ml solution of 40% methanol, 10% acetic acid, 50% 18 megohm-cm water for 30 minutes. 80ml of Colloidal Coomassie Blue stain was then mixed with 20ml of methanol and the gel was placed into the staining solution. The gel was allowed to stain for 1 hour.

The gel was placed in 25% methanol to destain the background for at least 1 hour. The background staining was decreased by repeating the destain step twice.

2.5.3 Western Blot Analysis

(Refer to Appendix for preparation of reagents and buffers)

For each gel, 4 pieces of filter paper were soaked in transfer buffer for 10 minutes. One piece of nitrocellulose membrane (Bio-Blot-NC, Costar Scientific) was also soaked in transfer buffer for the same amount of time to ensure efficient binding. Two pre-soaked sheets of filter paper were placed onto the platinum anode. The pre-wetted nitrocellulose membrane was then placed on top of the filter papers. The SDS-PAGE gel previously run was then carefully placed on top of the transfer membrane. The remaining 2 sheets of pre-soaked filter paper were placed on top of the gel. A test tube was rolled over the surface of the filter paper to exclude all air bubbles. The cathode was then carefully placed on to the stack and attached to a power supply. The power supply was turned on. Mini gels transfer for 60 minutes at 70 amps per gel. The filter paper and gel were then discarded. The following method was performed to detect the transferred proteins, employing the WesternBreeze® Chemiluminescent Kit (Invitrogen, WB7104). The kit included: blocking solution, secondary antibody diluent, ready-to-use tertiary antibody solution (anti-mouse), ready-to-use chemiluminescent substrate and wash solutions. The kit is stored at +4°C. (Refer to appendix for preparation of the solutions provided).

The membrane was placed in 10ml of the blocking solution in a large weighing boat for 30 minutes on a rotary shaker set at 1 rev./sec. After this blocking step, the blocking solution was decanted. The membrane was rinsed with 20ml of distilled water for 5 minutes, and then decanted. This was repeated once. The membrane was then incubated sequentially with 10 ml of positive control sheep colostrum (primary antibody) at a dilution of 1 in 50 with blocker (prepared in 2.4.1), 10ml of secondary antibody for 1 hour and 10ml of ready-to-use tertiary antibody for 30 minutes. The membrane was washed three times for 5 minutes with 20ml of Antibody wash between each incubation. The membrane was subsequently rinsed with 20ml of distilled water for 2 minutes. This was repeated twice.

The membrane was placed on a clean sheet of transparent plastic. Using a clean pipette, 2.5ml of the Chemiluminescent substrate was evenly applied to the surface of the membrane. The reaction was left to develop for 5 minutes. The excess Chemiluminescent substrate solution was blotted from the membrane surface with

filter paper. The membrane was covered with another piece of clean transparent plastic to prepare a membrane sandwich for luminography. An x-ray film (Amersham Pharmacia Biotech UK Ltd.) was exposed to the membrane sandwich for 15 seconds.

2.5.4 Determination of protein concentration

Protein concentrations in samples were determined using the BCA Protein Assay Reagent Kit (PIERCE, Rockford, USA). The BCA Protein Assay Reagent Kit includes: **BCA Reagent A;** 500ml, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, **BCA reagent B;** 25ml, containing 4% cupric sulphate, **Albumin Standard Ampules, 2 mg/ml;** 10 x 1ml ampules containing bovine serum albumin (BSA) at a concentration of 2.0 mg/ml in 0.9% saline and 0.05% sodium azide. The kit was stored at room temperature.

Protein standards from the stock solution of BSA, 2 mg/ml were prepared. BSA standard was diluted in PBS. Samples were prepared and diluted in PBS if required. 25µl volumes of each standard or sample were pipetted into the appropriate microtitre plate wells, using two wells per sample and two wells per BSA Protein standard (see table 2.3). 25µl of PBS was pipetted into each well of column 1 as the reagent blank wells.

The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B eg. 10ml Reagent A + 0.2ml Reagent B. This is stable for one day when stored at room temperature.

200µl/well of working reagent was added to all wells including the reagent blank wells, and mixed on a shaker. The plate was then covered with a plate sealer and incubated for a minimum of 30 minutes at 37°C. The plate was allowed to cool to room temperature and then the absorbance was measured at 562nm on a plate reader (DYNEX Technologies, UK).

	1	2	3	4	5	6	7	8	9	10	11	12
A		BSA 2mg/ml	BSA 2mg/ml	SAMPLE	SAMPLE							
B		BSA 1mg/ml	BSA 1mg/ml	SAMPLE	SAMPLE							
C		BSA 0.5mg/ml	BSA 0.5mg/ml	SAMPLE	SAMPLE							
D		BSA 0.25mg/ml	BSA 0.25mg/ml	SAMPLE	SAMPLE							
E		BSA 0.13mg/ml	BSA 0.13mg/ml	SAMPLE	SAMPLE							
G		BSA 0.063mg/ml	BSA 0.063mg/ml	SAMPLE	SAMPLE							
H		BSA 0.031mg/ml	BSA 0.031mg/ml	SAMPLE	SAMPLE							

B L A N K

Table 2.3 ELISA template for determination of protein concentration

2.6 Determination of IgE bearing cells by Fluorescence-activated cell sorter (FACS) analysis

Fluorescence-activated cell sorter (FACS) analysis was used to determine the percentage of IgE bearing cells (%IgE) present within all the 98 Scottish Blackface lambs.

In order to remove the red blood cells, 1ml whole blood was mixed with 4ml of lysing solution (Tris-NH_4Cl) (previously warmed to 37°C) in a 15ml centrifuge tube and kept at room temperature until haemolysis was observed. The lysed red blood cells were immediately centrifuged at 1500 r.p.m for 8 minutes at 4°C. The supernatant was decanted and cells were resuspended in FACS buffer (5% Foetal Calf Serum, 0.2 % Sodium Azide [NaN_3] in Phosphate Buffered Saline [PBS]). Cells were washed three times in FACS buffer at 4°C.

100µl of 2F1 αIgE monoclonal antibody 1:50 dilution in FACS buffer was added to 100µl of previously prepared cells, in a 96-well U-bottomed plate and incubated for 30 minutes at 4°C on ice in the dark. Positive and isotype controls were included for each animal. Cells were incubated with monoclonal anti-ovine Ig light chain (VPM8, Moredun Research Institute, Edinburgh, Batch no. 61098), 1: 800 dilution in FACS buffer to be used as a positive control and cells were incubated with mouse immunoglobulin IgG1 (the same isotype as 2F1)(SIGMA, Germany, M-5284) diluted at 1:200 dilution in FACS buffer to be used as an isotype control.

The cells were then washed three times in 200µl of FACS buffer and incubated with 25µl of α-mouse IgG1 Alexa Fluor conjugate antibody (Molecular Probes, Oregon, USA, A11006) diluted 1:500 in FACS buffer for 30 minutes on ice, in the dark. The cells were washed three times in 200µl of FACS buffer and then once in 200µl of PBS-NaN_3. Finally, the supernatant was decanted and the stained cells were stored at 4°C in 200µl 1% paraformaldehyde until analysed by the flow cytometer, 24 hours later. Data acquisition and analysis was performed with a two laser four-colour FACS Calibur flow cytometer and Cell Quest software (Becton Dickinson, Mountain View, California). 10,000 cells were acquired for each sample. Alexa Fluor fluorescence was analysed in the channel FL1.

2.7 Statistical Analysis

Results were expressed as group means and s.e.m's. Differences between groups of lambs were compared using a two-tailed Student's t-test. Correlations between two variables were calculated using Spearman's rank correlation to see whether the two variables to be tested covary. The analyses were carried out using Minitab Statistical Software, Release 13.30. $P<0.05$ was considered statistically significant in both statistical tests.

Chapter 3

Purification and characterisation of *Teladorsagia circumcincta* third stage (L_3) larval antigen

3.1 INTRODUCTION

Gastrointestinal nematode parasite infections are characterised by a marked increase in IgE antibody in the serum of infected hosts (Jarrett and Miller, 1982). It is yet to be established how significant this antibody is in terms of protective immunity although it has been suggested that IgE may have a fundamental and protective function against some helminth parasites (Ahmad *et al.* 1991, Verwaerde *et al.*, 1987). A number of studies have identified antigenic determinants that may be significant in the development of immunity to nematode parasites, and many of these are glycoconjugates that are secreted and/or bound to the surface of the parasite (Dell *et al.*, 1999). Examples of these antigens include the immunodominant surface antigens on the larvae of *H. contortus* (Bowles *et al.*, 1995), *Dictyocaulus viviparus* (Gilleard *et al.*, 1995) and *Toxocara canis* (Maizels *et al.*, 1990). However, relatively few allergens generating specifically IgE responses have been identified and characterised. These include the polyprotein allergens of *Ascaris spp* (Muto *et al.*, 2001) and *Toxocara canis* (Yahiro *et al.*, 1998), the surface associated glycoproteins of *Brugia malayi* and *Wuchereria bancrofti* (Paxton *et al.*, 1993), tropomyosin from *Onchocerca volvulus* (Jenkins *et al.,* 1998) and the major membrane allergens of *schistosomula* (Santiago *et al.*, 1998). As discussed in the general introduction, previous studies on the immune response to *T.circumcincta* in sheep have shown that nematode infections are also characterised by a marked IgE antibody response which is directed predominantly against the infective third stage larvae (L_3) but not against the adult (L_5) worms (Huntley *et al.* 1998a). Subsequent investigations using electron microscopy have shown that the IgE reactivity seems to be confined to the surface of the infective L_3 larvae including its sheath as demonstrated in the ultra structure immuno electron micrograph in figure 3.1 (Huntley *et al.*, 2001). These findings have led to the identification of a specific *T. circumcincta* L_3 antigen that plays a key role in the induction of local IgE antibody production. Previous studies with the glycoprotein from *H. contortus* L_3 larvae have demonstrated that vaccination with purified larval surface antigen from *H.contortus* induced a significant response and showed that the surface larval antigens may have potential as vaccine components (Jacobs *et al.*, 1999). It is not

yet established whether the *T. circumcincta* L_3 surface antigen is comparable in function or vaccine potential to the *H.contortus* L_3 surface glycoprotein.

An initial objective was to purify the high molecular weight (140-150kDa) surface allergen from *T.circumcincta*, and further analyse this protein in terms of its physicochemical characteristics and antigenic reactivity. For the latter, an ELISA was developed for the detection and quantification of IgE, IgA and IgG antibodies to this protein in blood. These assays formed the basis in determining if the generation of these circulating antibodies relate to the development of immunity in lambs to *T.circumcincta*, and therefore their value as markers of responsiveness. Although previous work had suggested that IgE responses were mainly confined to the infective larval L_3 stage (Huntley *et al.*, 2001), the present work has further investigated the IgE responses to the infective L_3, pre-adult (L_4) and adult (L_5) stages of *T.circumcincta*. Finally, the specificity of the IgE response was evaluated, by investigating whether cross-reactive IgE antibodies are generated during infection to *T.circumcincta*, against other species of infective larval nematode antigens.

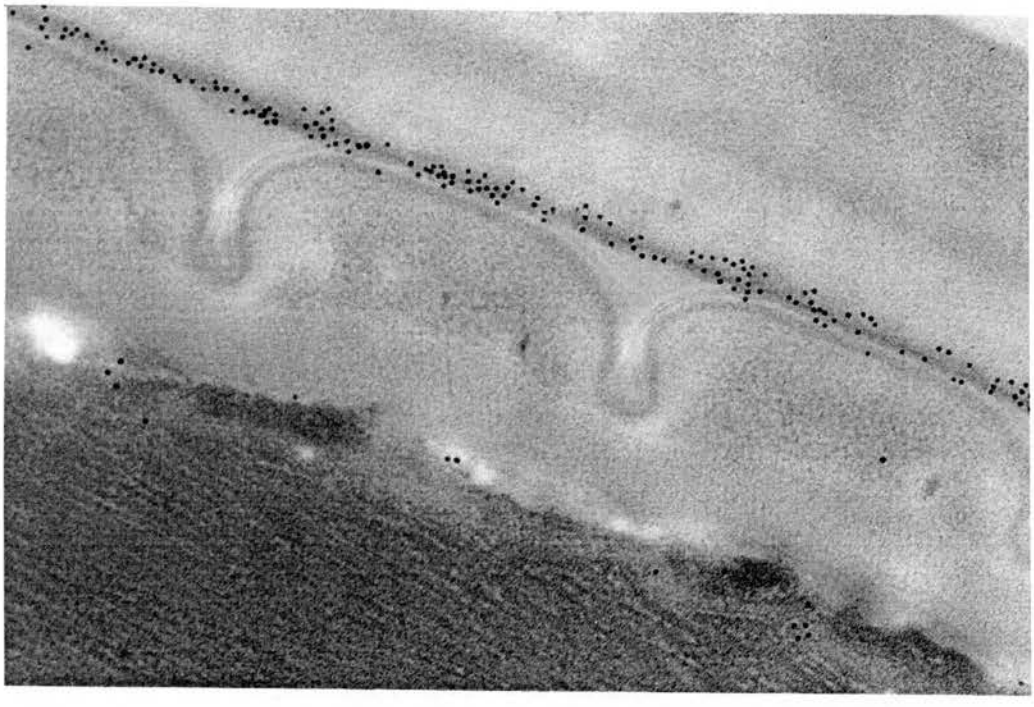


Figure 3.1 Electron micrograph demonstrating the surface of a *T. circumcincta* infective L_3 larvae and the localisation of the IgE reactive allergen (Brennan G, Queens University, Belfast) as shown by the immunogold particle labelling.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of whole soluble extract from different species of infective L_3 larvae

Whole larval antigen from several different species of infective L_3 larvae was prepared using the method described in 2.2.8. The species of parasite used were *T.circumcincta*, *T.colubriformis*, *H.contortus*, *N.battus,* and *C.curticei*. The origins of these parasites are described in detail in 2.2.4.

3.2.2 Preparation of crude soluble extract from *T.circumcincta* L_4 larvae and L_5 larvae

Whole larval antigen from L_4 and L_5 *T.circumcincta* larvae was prepared using the method described in 2.2.8.

3.2.3 SDS-PAGE analysis of crude *T.circumcincta* L_3 antigen to determine its molecular weight

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Samples of whole soluble *T.circumcincta* L_3 extract were treated with non-reducing sample buffer and heated to 100°C in a water bath for 3 minutes. Since preliminary work had indicated that the allergen stained poorly, a relatively high loading of 50μg was applied to one track of the gel. Mark 12 molecular weight markers (10μl) (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was stained with Colloidal Coomassie blue stain as described in 2.5.2.

3.2.4 Western Blot analysis to determine IgE binding components of *T.circumcincta* L_3 antigen

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Samples of whole soluble *T.circumcincta* L_3 extract were either treated with non-reducing sample buffer or reducing sample buffer and heated to 100°C for 3 minutes before being loaded on to the gel at 5μg. Mark 12 markers (10μl) (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was blotted and probed with sheep colostrum (2.4.1) using the method described in

2.5.3. The secondary antibody employed in this procedure was the anti-IgE monoclonal, 2F1 (Windon *et al.*, Veterinary Immunology and Immunopathology, In Press) used at a previously optimised concentration of 1 in 1000 in blocking buffer.

3.2.5 Purification of *T.circumcincta* whole larval antigen by gel permeation chromatography

3.2.5.1 Preparation of samples

Stored infective third stage *T.circumcincta* larval antigen (1ml) (prepared as shown in 2.2.7) was removed from –20°C and allowed to thaw. The larval antigen was then centrifuged for approximately 5 minutes at 10,000rpm using a microfuge (Mini Spin Plus, Eppendorf, Germany). The supernatant was then drawn up in a 1ml syringe ready to fractionate as subsequently described.

3.2.5.2 Fractionation of T.circumcincta *soluble extract by size exclusion chromatography*

It was established that the *T.circumcincta* L_3 larval antigen has a molecular weight of approximately 150kDa and so the optimum column chosen was a Sephacryl 300 (S300)(1.5cm X 30cm, 17-0599-101, Pharmacia Biotech, UK). Using High Performance Liquid Chromatography (HPLC) (Waters, Milford, U.S.A), 1ml of *T.circumcincta* whole larval antigen was applied to a S300 column in PBS/0.1% sodium azide (NaN_2) at a flow rate of 1ml per minute. Fractions (1ml) were collected and the OD at 280nm was monitored using a chart recorder. 100 fractions were collected and retained for further analysis.

3.2.5.3 Identification of fractions by dot blot analysis

Each fraction collected was dotted on to nitrocellulose membrane (2µl) (Bio-Blot-NC, Costar Scientific). 5µg of *T.circumcincta* whole soluble extract prepared in 2.2.7 was also applied to the membrane (2µl) and used as a positive control. The nitrocellulose was left to air dry and then blocked for 30 minutes in PBS/5% skimmed milk powder (Marvel, Premier Brands Ltd, UK). The chemiluminescent Western Blotting Immunodetection System (Western Breeze, Invitrogen, WB7104) was employed for the remainder of the method as described in 2.5.3. The secondary

antibody used in this procedure was anti-IgE monoclonal, 2F1 at a 1 in 1000 dilution in blocking buffer.

3.2.6 Purification of *T.circumcincta* whole larval antigen by affinity chromatography

Further purification of S300 *T.circumcincta* was carried out using affinity chromatography. Firstly, using High Performance Liquid Chromatography (HPLC) (Waters, Milford, U.S.A), 1ml of previously prepared S300 purified *T.circumcincta* L_3 antigen was applied to a Superose 6 column (S6 - Pharmacia Biotech, UK) in PBS/0.1% sodium azide (NaN_2) at a flow rate of 0.5ml per minute. Fractions (1ml) were collected and the OD at 280nm was monitored using a chart recorder. Fractions of interest, as tested by dot blot (see 3.2.5.3) were collected.

Two New Zealand rabbits were inoculated with 15μg of S6 purified *T.circumcincta* L_3 antigen together with 50μg of Quil A adjuvant (Sigma, St Louis, MO, USA), three times over a period of three months. Antiserum (5ml) collected from the rabbits was passed through a HiTrap Protein A sepharose column (Amersham Pharmacia, UK) at 1ml per minute equilibrated in 20mM sodium phosphate buffer, pH7.5. The IgG fraction was eluted with 0.1M glycine at pH 2.5 then neutralised immediately with 30μl of 1M Tris. The IgG fraction collected (containing the anti-S6 purified *T.circumcincta* allergen) was dialysed overnight against coupling buffer (0.1M $NaHCO_3$, 0.5 M NaCl, pH8.3) changing the dialysate three times.

Sepharose-4B (0.75g) (Amersham Pharmacia, UK) was equilibrated in 1mM cold hydrochloric acid (HCl) and washed on a sintered glass filter funnel with 400ml of the HCl solution. After this washing step the gel was pulled dry under vacuum. The dialysed IgG ligand was then mixed with the gel (1:2 gel to ligand) and rotated overnight at 4°C. The gel suspension was packed into a 2ml bed volume column (Amersham Pharmacia, UK) and washed with 10 column volumes of 1M ethanolamine to block remaining active groups. To wash away excess adsorbed protein the gel was washed with coupling buffer followed by 0.1M acetate buffer, pH4 containing 0.5M NaCl followed again by coupling buffer. The column was equilibrated in 50mM Tris/HCL, pH8. Prepared whole *T.circumcincta* L_3 antigen

(10ml) (2.7.1) (0.6mg/ml) was applied to the column at a flow rate of 1ml per minute. The affinity purified *T.circumcincta* L_3 antigen was eluted from the column using 0.1M glycine, pH 2.5 and immediately neutralised with 30µl of 1M tris. The fractions collected were stored at –20°C for subsequent analysis.

3.2.7 SDS-PAGE and IgE western blot analysis of whole soluble extract and S300 purified soluble extract of *T.circumcincta* L_3 antigen

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Samples of whole soluble extract and S300 purified soluble extract were treated with non-reducing sample buffer and loaded on to the gel at 50µg. Mark 12 markers (10µl) (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was stained with Colloidal Coomassie blue stain as described in 2.5.2. For western blot analysis, SDS-PAGE was performed as described in 2.5.1. Samples of whole soluble extract and S300 purified soluble extract were treated with non-reducing sample buffer, heated to 100°C for 3 minutes and 5µg was applied to each track. Markers (10µl) were included on the gel (Mark 12, Invitrogen, Carlsbad, CA). Following electrophoresis, the proteins were transferred to nitrocellulose membrane and probed with sheep colostrum (2.4.1) using the method described in 2.5. The secondary antibody used in this method was the anti-IgE monoclonal, 2F1 at a dilution of 1 in 1000.

3.2.8 Determination of carbohydrate on S300 purified *T.circumcincta* L_3 antigen by periodate treated and non-periodate treated ELISA

The preparations of reagents and buffers for these studies are shown in the appendix.

To determine if carbohydrate epitopes were important for IgE antibody response, an ELISA was carried out to demonstrate if there is a reduction in IgE immunoreactivity in the S300 purified *T.circumcincta* L_3 antigen after periodate treatment, and the method was adapted from Afferni *et al*, 1999 and Woodward *et al*, 1985. Periodate oxidises carbohydrates therefore prior treatment will reduce OD's of IgE reactive carbohydrate epitopes involved. Employed samples contained high IgE antibody levels and positive and negative controls were included. Sets of ELISA

wells were exposed to varying concentrations of periodate (0-50mM) to determine at which concentration of periodate the IgE immunoreactivity of the S300 purified *T.circumcincta* L_3 antigen reduces. The details of the assay were as follows: -

An ELISA plate (M129B, Dynatech Lab Ltd) was incubated overnight at 4°C with S300 purified *T.circumcincta* L_3 antigen at 1μg/ml (50μl/well) in carbonate/bicarbonate buffer, pH 9.6. The antigen was then discarded and the plates were washed twice in PBS T20. The wells were blocked with PBS/Tween 80 + NaCl and incubated for 30 minutes at room temperature. The plates were then washed twice in PBS T20. The controls and the template of the microtitre plate are shown in table 3.1 and 3.2 respectively.

ELISA plate column	Periodate treatment	Buffers (Acetate/borohydride)	Sheep sera
1 (neg)	No	No	No
2 (neg)	Yes	Yes	No
3 (pos)	No	No	Yes
4 (pos)	Yes	Yes	Yes

neg = Negative Control

pos = Positive control

Table 3.1 Table to show the positive and negative controls used in this periodate treatment

	1	2	3	4	5 ← periodate → 9					10	11	12
A	Neg	Neg	Pos	Pos	5mM	10mM	15mM	20mM	50mM			
B	↓	↓	↓	↓	↓	↓	↓	↓	↓			
C	↓	↓	↓	↓	↓	↓	↓	↓	↓			
D	↓	↓	↓	↓	↓	↓	↓	↓	↓			
E	↓	↓	↓	↓	↓	↓	↓	↓	↓			
F	↓	↓	↓	↓	↓	↓	↓	↓	↓			
G	↓	↓	↓	↓	↓	↓	↓	↓	↓			Pos
H	▼	▼	▼	▼	▼	▼	▼	▼	▼			▼

Table 3.2 ELISA plate template for the periodate treatment of S300 purified *T.circumcincta* L_3 antigen.

Columns 2,4,5,6,7,8 and 9 were then rinsed with 50mM sodium acetate buffer (pH 4.5) prior to exposure with various concentrations (5mM to 50mM) of periodate.
Columns 1,3 and 12GH were rinsed with PBS T20.
Columns 2,4,5,6,7,8 and 9 were then exposed to periodate (50µl/well).
2 AB = 5mM periodate (sodium) with acetate buffer
2 CD = 10mM periodate (sodium) with acetate buffer
2 EF = 15mM periodate (sodium) with acetate buffer
2 GH = 20mM periodate (sodium) with acetate buffer
4 = 15mM periodate (sodium) with acetate buffer
5 = 5mM periodate (sodium) with acetate buffer
6 = 10mM periodate (sodium) with acetate buffer
7 = 15mM periodate (sodium) with acetate buffer
8 = 20mM periodate (sodium) with acetate buffer
9 = 50mM periodate (sodium) with acetate buffer

Columns 1,3 and 12 GH were exposed to PBS T20 (50µl/well). The plate was incubated with the periodate treatment and PBS T20 for 1 hour at room temperature in the dark. The wells that had been periodate treated were then rinsed briefly with 50mM sodium acetate buffer and then incubated with 50mM sodium borohydride in PBS for 30 minutes at room temperature (50µl/well) (this included column 4 of the plate, see table 3.2). This treatment reduces the aldehyde groups generated by periodate oxidation to alcohols and prevents non-specific cross-linking of antibody to antigen. The columns without periodate treatment were rinsed with PBS T20 for a further 30 minutes at room temperature (50µl/well). The plate was then washed five times with PBS T20 and then incubated with either of the following: -

1. Sheep sera previously shown to have a relatively high IgE titre diluted at 1/10 dilution with PBS T20.

   Columns 3,4,5,6,7,8, and 9
2. Sheep colostrum (see 2.4.1) at a dilution of 1/10 in PBS T20.

   Column 12 Row GH
3. PBS T20

   Columns 1 and 2

The columns on the ELISA plate were incubated with one of the above options at 50µl/well at room temperature for 1 hour.

The plate was washed five times and incubated with the anti IgE 2F1 monoclonal antibody at 1/1000 dilution with PBS T20 for 1 hour at room temperature (50µl/well). The plate was then washed five times with PBS T20. All wells were subsequently incubated with goat anti-mouse biotinylated antibody (DAKO Ltd., Denmark, EO433) at 1/1000 dilution with PBS T20 for 1 hour at room temperature (50µl/well). The plate was washed with PBS T20 five times and incubated with Streptavidin HRP conjugate (DAKO Ltd., Denmark, PO397) at 1/2000 dilution with PBS T20 for 30 minutes at room temperature (50µl/well). The plate was washed five times with PBS T20. The wells were finally incubated with OPD peroxidase substrate (SIGMA FAST tablet set)(50µl/well) until colour had developed enough to give a reasonable OD at 492nm. The reaction was stopped by the addition of 2.5M H_2SO_4 (25µl/well). The plate was read at 492nm on a plate reader (Dynex Technologies INC, UK).

3.2.9 Glycoprotein and Lipoprotein analysis of S300 purified *T.circumcincta* L_3 antigen

To determine whether the high molecular weight antigen of *T.circumcincta* could be characterised as a glycoprotein or lipoprotein, the protein was stained in SDS-PAGE gels and agarose gels respectively, with the following commercial staining kits: - a) GelCode® Glycoprotein Staining Kit (PIERCE, Rockford USA), b) Pro-Q™ Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes, Oregon, USA, P-21855) and c) Nile Red to determine lipoproteins (10µg/ml acetone, Sigma, St Louis, MO). The preparations of the reagents are described in the appendix. All procedures were as described by the manufacturer's instructions as follows: -

a) Discontinuous SDS-PAGE was carried out as described in 2.5.1. The samples added to the gel were as follows: - Lane 1 was loaded with Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA,); Lane 2 with reconstituted positive control; Lane 3 with reconstituted negative control; Lane 4 with S300 *T.circumcincta* L_3 antigen diluted to a 1mg/ml solution with reducing buffer and previously heated to 100°C for 3 minutes; Lane 5, no sample; Lane 6 was loaded with the positive control;

Lane 7 with the negative control and Lane 8 was loaded with S300 *T.circumcincta* L_3 antigen diluted to a 1mg/ml solution with non-reducing buffer and previously heated to 100°C for 3 minutes. Each sample (10µl) was applied to each track of the gel. Following electrophoresis, the gel was fixed by completely immersing it in 100ml of 50% methanol for 30 minutes. The gel was then washed by gently agitating with 100ml of 3% acetic acid for 10 minutes. This step was repeated once. The gel was then transferred to 25ml of Oxidation Solution and gently agitated for 15 minutes. The gel was washed by gently agitating with 100ml of 3% acetic acid for 5 minutes. This step was repeated two more times. The gel was then transferred to 25ml of GelCode® Glycoprotein Staining Reagent and gently agitated for 15 minutes, transferred to 25ml of Reduction Solution and gently agitated for 5 minutes. The gel was washed extensively with 3% acetic acid and then with dH_2O. Glycoproteins were seen as magenta bands. The gel was stored in 3% acetic acid and a photograph of the gel was taken.

b) Proteins were separated by standard SDS-PAGE (2.5.1). The S300 *T.circumcincta* L_3 antigen sample was diluted to 50µg/ml with reducing buffer and non-reducing buffer and heated to 100°C for 3 minutes. The diluted sample (10µl) was applied to each track of the gel. The template of the gel was as follows: - Lane 1 and Lane 4 were loaded with 10µl of the Candy Cane glycoprotein standard (provided in the kit); Lane 2 was loaded with the antigen sample with non-reducing sample buffer and Lane 3 was loaded with the antigen sample with reducing sample buffer. Following electrophoresis, the gel was fixed by immersing it in 75-100 ml of Fix Solution (provided in the kit) and incubated at room temperature with gentle agitation for 45 minutes. The gel was then washed in 50 ml of Wash Solution with gentle agitation for 10 minutes. This step was repeated once. The gel was then incubated in 25 ml of Oxidising Solution (provided in the kit) with gentle agitation for 30 minutes. The gel was washed in 50 ml of Wash Solution with gentle agitation for 5-10 minutes. This step was repeated twice more.

Fresh Pro-Q Emerald 300 Staining Solution was prepared by diluting Component A 50-fold into Component B. The gel was then incubated in the dark in 25 ml of Pro-Q Emerald 300 Staining Solution while gently agitating for 120 minutes. The signal was seen after 20 minutes and maximum sensitivity was reached at about 120

minutes. The gel was then washed with 50 ml of Wash Solution at room temperature for 15 minutes. This wash was repeated once for a total of two washes. The stained glycoproteins were visualised by using a 300nm UV transilluminator. A photograph was taken of the gel.

c) This final method was carried out to determine if lipoproteins were present within the composition of S300 *T.circumcincta* L_3 antigen. Nile Red solution (20µl) (10µg/ml acetone, Sigma, St Louis, MO) was added to 13 x 100mm glass test tubes and the acetone was allowed to evaporate. 50µl of samples at a known concentration were added to the tubes and swirled by hand for approximately 5 seconds. Two samples were employed in this procedure: a lipoprotein standard (Lipoproteins, SIGMA, Germany, L3626) used as a positive control loaded at a 500µg/ml concentration and the high molecular weight S300 purified antigen loaded at the same concentration. 5µl of a 30% weight/volume (w/v) sucrose solution was then added to the tubes and the samples were loaded onto the agarose gel.

A 0.6% w/v solution of agarose in 50ml of 50mM barbital buffer, pH 8.6 was prepared by heating to 85°C. After allowing the agarose solution to cool, a 25ml aliquot was added to a taped running tray of a Minne Horizontal Agarose Submarine unit containing an 8 well comb, 1mm thick. After allowing the gel to form, the running tray was placed in the electrophoretic chamber filled with 50mM barbital buffer, pH 8.6. The gel was loaded with all the samples including a positive control and electrophoresed for 2 hours at 56 volts at room temperatre. The gel, still in the running tray, was then viewed for Nile Red fluorescence under ultra violet light. The agarose gel was separated from the running tray and photographed under ultra violet light with a Polaroid camera.

3.2.10 Characterisation of S300 purified L_3 *T.circumcincta* antigen using Mass Spectrometry - Tryptic Peptide Mapping from SDS-PAGE gels

3.2.10.1 Preparation of samples

The high molecular weight S300 *T.circumcincta* L_3 antigen shown on a Colloidal Coomassie stained gel (3.2.7) was excised using a sterile scalpel. To achieve adequate spectrometric analysis, all traces of Coomassie Blue dye were removed. This was carried out by firstly mixing the gel piece for 10 minutes in 500µl 10%

acetic acid solution. The solution was subsequently removed and the gel was washed thoroughly with distilled water. The gel piece was washed with 500µl acetonitrile followed by methanol for 20 minutes each time. Between the changes of solvents, the gel piece was washed with distilled water. To destain the gel completely, 1ml of formic acid:distilled water:isopropanol (1:3:2, v,v,v) was added and mixed for 30 minutes to 3 hours until the gel turned colourless.

3.2.10.2 In-Gel Reductive Alkylation

250µl 100mM ammonium bicarbonate/50% acetonitrile was added to the gel in a 1.5ml eppendorf and washed for 30 minutes on a suspension mixer. The supernatant was then discarded. The gel piece was dried using a vacuum centrifuge for approximately 30 minutes to allow for complete saturation with reducing/alkylating reagents. 250µl 10mM dithiothreitol in 100mM ammonium bicarbonate was added to the eppendorf and incubated at 60°C for 30 minutes to allow reduction to occur. The supernatant was then discarded. The sample was allowed to cool to room temperature. 250µl 100mM idoacetic acid in 100mM ammonium bicarbonate was added to the eppendorf and incubated in darkness at room temperature for 30 minutes to allow alkylation to occur. The supernatant was discarded and 250µl of 100mM ammonium bicarbonate/50% acetonitrile was added to the piece of gel and washed for 30 minutes by vigorous shaking on a suspension mixer. The supernatant was discarded and the gel piece dried using a vacuum centrifuge for approximately 30 minutes to allow for complete saturation with enzyme.

3.2.10.3 Trypsin Digestion

The content of one glass vial containing 20µg trypsin (Promega sequence grade porcine trypsin) was rehydrated with 50µl resuspension buffer (Promega). 98µl 100mM ammonium bicarbonate plus 2µl resuspended enzyme solution was added to the dry gel piece and incubated at 37°C overnight.

3.2.10.4 Extraction of Peptides

The supernatant from the eppendorf was removed and placed in another labelled 1ml eppendorf so that any peptides that had leached from the gel were retained.

Peptides were extracted from the gel by adding 100µl 60% acetonitrile/0.1% trifluoroacetic acid to the eppendorf and was shaken vigorously on a suspension mixer for 60 minutes. The supernatant was removed and combined with the other respective supernatant. The extraction procedure was repeated again and all supernatants were combined (total volume: 300µl). All of the solvent was evaporated using a vacuum centrifuge plus heat for a few hours. The result was a small pellet/glaze on the bottom of a dry eppendorf. The sample was stored at –70°C until it was analysed by mass spectrometry.

3.2.10.5 Mass Spectrometry

The sample was analysed using an Applied Biosystems Voyager DE-PRO Matrix Assisted Laser Desorption Ionisation – Time of Flight (MALDI-TOF) Mass Spectrometer. Using the manufacturer's guidelines a standard peptide mass fingerprint covering the 700-50000 Daltons range was obtained in the reflectron mode. Once a mass spectrum was obtained for the protein sample of interest, the peptide mass information provided was entered into the MS-FIT database software. The MS-FIT database software works by correlating the mass spectrometry data with a protein in a sequence database which best fits the data.

3.2.11 N-Terminal Amino Acid Sequencing

Following electrophoresis (see 2.5.1), the SDS-PAGE gel was carefully taken off the cassette and transferred by western blot (see 2.5.3) to PVDF (Immobilon) membrane (Millipore, UK). The template of the SDS-PAGE gel was as follows; Lane 1 was loaded with 10µl of Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA) and Lane 2 was loaded with 10µl of S300 *T.circumincta* L_3 antigen containing 50µg of protein. The blot was then stained with Coomassie Blue Stain (SIGMA, Germany) and the S300 *T.circumcincta* L_3 protein band of interest was excised. The excised band was then destained with 50% methanol/distilled water for 10 minutes then washed thoroughly with distilled water. The sample was then loaded into a PVDF cartridge on a Procise 494 Protein Sequencer (Applied Biosystems). The sequencer then automatically carried out Edman Degradation. Analysis of the sample was then undertaken by Reversed-phase HPLC (C-18) and

comparisons were made with a standard mixture of phenylthiohydantoin amino acids (injected onto the column as the first cycle). Data analysis software was then used to determine amino acid residues.

3.2.12 SDS-PAGE and IgE Western Blot analysis of whole soluble infective L_3 antigen from six different species of sheep parasite

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Samples of whole soluble extract from *T.circumcincta*, *T.colubriformis*, *H.contortus*, *N.battus* and *Cooperia curticei* were treated with non-reducing sample buffer, heated to 100°C for 3 minutes and applied to each track of the gel at 50µg. Mark 12 markers (10µl) (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was stained with Colloidal Coomassie blue stain as described in 2.5.2. For western blot analysis, SDS-PAGE was performed as described in 2.5.1. Samples of whole soluble extract from the above six species of parasite were treated with non-reducing sample buffer, heated to 100°C for 3 minutes and applied to each track of the gel at 5µg. Markers (10µl) were included on the gel (Mark 12, Invitrogen, Carlsbad, CA). Following electrophoresis, the proteins were transferred to nitrocellulose membrane and probed with sheep colostrum (2.4.1) using the method described in 2.5. The secondary antibody used in this method was the anti-IgE monoclonal, 2F1 at a dilution of 1 in 1000.

3.2.13 SDS-PAGE and IgE Western Blot analysis of whole soluble infective L_3, L_4, and L_5 larval antigen from *T.circumcincta*

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Samples of whole soluble extract of L_3, L_4, and L_5 *T.circumcincta* were treated with non-reducing sample buffer, heated to 100°C for 3 minutes and applied to each track of the gel at 50µg. Mark 12 markers (10µl) (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was stained with Colloidal Coomassie blue stain as described in 2.5.2. For western blot analysis, SDS-PAGE was performed as described in 2.5.1. Samples of whole soluble extract of L_3, L_4, and L_5 *T.circumcincta* were treated with non-reducing sample buffer, heated to 100°C for 3 minutes and applied to each track of the gel at 5µg. Markers

(10μl) were included on the gel (Mark 12, Invitrogen, Carlsbad, CA). Following electrophoresis, the proteins were transferred to nitrocellulose membrane and probed with sheep colostrum (2.4.1) using the method described in 2.5. The secondary antibody used in this method was the anti-IgE monoclonal, 2F1 at a dilution of 1 in 1000.

3.2.14 Western Blot analysis to determine IgE, IgA and IgG binding components of *T.circumcincta* L_3 antigen

Discontinuous SDS-PAGE was conducted as described in 2.5.1. Three samples of whole soluble *T.circumcincta* L_3 extract were treated with non-reducing sample buffer, heated to 100^0C for 3 minutes and applied to each track of the gel at 5μg. 10μl of Mark 12 markers (Invitrogen, Carlsbad, CA) were also applied to the gel. Following electrophoresis, the SDS-PAGE gel was blotted using the method described in 2.5. Prior to using the Western Breeze Kit (2.5.3), the nitrocellulose membrane was cut into strips (each strip containing the blotted antigen of interest) so that the antigen could be tested individually for IgE, IgA and also IgG binding components. The secondary antibodies employed in this procedure were anti-IgE 2F1 monoclonal antibody used at a concentration of 1 in 1000, anti-ovine IgA (Serotec, Oxford, UK) and anti-ovine IgG antibodies (VPM6 ascites, MRI, Edinburgh) both used at 1 in 2000 dilution.

3.2.15 Development of a parasite specific antibody ELISA

A parasite specific ELISA was adapted from Huntley *et al.*, 2001 to detect and quantify circulating levels of IgE, IgA and IgG antibodies that have previously been shown to recognise the S300 purified *Teladorsagia circumincta* 3rd stage larval surface antigen (3.3.12). Ten random serum samples, across the grazing season, from Greyface x Suffolk lambs that were naturally infected with *T.circumcincta* (see 2.1.1) were tested for three different parasite specific antibody isotypes using the method described in 2.4.2. Previous titration ELISA studies were carried out to determine the optimal dilutions of primary and secondary antibodies to use in this assay (unpublished observations). The anti-IgE 2F1 monoclonal secondary antibody was employed at a dilution of 1 in 1000 and the anti-IgA and anti-IgG secondary

antibodies were used at a dilution of 1 in 2000. The concentration of serum for detecting IgG antibodies was 1 in 200 whereas the detection of IgA and IgE, employed diluting the sera at 1 in 10. Preliminary investigations showed that IgE, IgA and IgG levels increased over the grazing season in all the animals, although the titres of IgE antibody were low in comparison to the IgA and IgG levels (unpublished observations).

3.2.16 Influence of IgG and IgA antibodies on IgE antibody detection

The results shown in 3.3.12 demonstrated that the S300 purified *T.circumcincta* L_3 antigen of interest is recognised by IgA and IgG antibodies as well as IgE antibodies and it was thought that the low levels of parasite specific IgE levels found in 3.2.15 were perhaps due to the IgA and IgG antibodies competing with IgE for the same epitope on the antigen of interest. It was hypothesised that if the IgE antibodies could be specifically extracted from the sera, then the influence of competing IgG and IgA antibodies (probably present in excess of IgE) would be abrogated. The IgE was specifically extracted from the sera by solid phase immunoaffinity employing the anti-IgE monoclonal 2F1, as described below.

3.2.16.1 Generation of 2F1-CNBr Sepharose beads

The anti-IgE 2F1 monoclonal antibody was initially affinity purified, and then bound to the CNBr sepharose 4B (17-0981-01, Amersham Biosciences, Bucks, UK). This was achieved using the HPLC equipment (Waters, Milford, USA). The sample of 2F1 tissue culture supernatant (100ml) was injected on to a super loop and then run on to a rat anti-IgG column (SIGMA, Oxford, UK) at a flow rate of 0.5ml per minute. Glycine (0.1M), pH 2.5 was added to the column to elute the 2F1. The fractions were collected and neutralised immediately with 1M tris (30µl). Purified 2F1 yielded 1mg of protein. Once the purified 2F1 was attained, it was tested by ELISA (2.4.2) for anti-IgE reactivity before it was bound to CNBr sepharose beads as described by the manufacturer. The results of the ELISA demonstrated that the affinity purified 2F1 monoclonal retained its antibody activity and was highly reactive to ovine IgE (unpublished observations).

The affinity purified 2F1 was then dialysed overnight in coupling buffer, 0.5M sodium hydrogen carbonate ($NaHCO_3$), pH 8.3 containing 0.5M NaCl at 4°C and a PIERCE protein test (see 2.5.4) was performed to calculate the total protein content. A total of 4.7mg of 2F1 monoclonal antibody was coupled to 2.5ml of CNBr gel according to the manufacturer's instructions. After coupling, non-reacted groups on the medium were blocked by keeping the coupled medium in 0.1M Tris/HCl, pH 8.0 at room temperature on a rotary mixer for a few hours. The coupled product was then washed with at least 3 cycles of high and low pH (0.1M acetate buffer, pH 4.0 and 0.1M Tris, pH 8.0, both containing 0.5M NaCl) with 5 gel volumes of each buffer per cycle. The gel was then placed in a universal with phosphate buffered saline (PBS) and 0.02% sodium azide (NaN_2) and stored at 4°C. An IgE ELISA 2F1-sepharose bead assay was then developed.

3.2.16.2 Extraction of IgE antibody

IgE was removed from sera, by incubating sera in 2F1-Sepharose 4B beads. By carrying out a number of trial procedures using sera with relatively high IgE levels (as detected by the ELISA described in 2.4.2), it was possible to define the quantity of 2F1-sepharose beads to employ in the assay, the volume of sera required, the volume of glycine necessary to elute the IgE from the 2F1-sepharose beads and the amount of tris needed to neutralise the sample. The outcome of these investigations established that 10µl of 2F1-sepharose beads and 25µl of sera were employed in the extraction procedure. The amount of glycine (0.1M, pH2.5) necessary to remove the IgE from the beads was 50µl and 2µl 1M tris was required to neutralise the sample (unpublished observations). PBS was added to the eluted IgE sera to give a final dilution of 1 in 10. A dot blot assay employed to determine the efficiency of this IgE extraction procedure demonstrated that the method was successful at extracting all the IgE immunoglobulins from the sera.

Following these preliminary investigations, ten different serum samples with relatively high IgE levels (as detected by the ELISA method described in 2.4.2) were processed to remove the IgE immunoglobulin with 2F1-sepharose 4B beads. The OD values of the extracted immunoglobulins and the original sera were compared by the IgE ELISA (3.2.15). Both the IgE eluted and the original serum samples were

assayed at a dilution of 1 in 10. The original sera and 2F1-sepharose extracted immunoglobulins were also assayed for the presence of IgG and IgA antibodies (3.2.15).

3.3 RESULTS

3.3.1 Characterisation of whole *T.circumcincta* L_3 antigen

Figure 3.2 demonstrates the protein bands present within the whole crude extract of *T.circumcincta* L_3 antigen (prepared in chapter 3.2.1). A number of protein bands were visualised within this sample as indicated by the arrows.

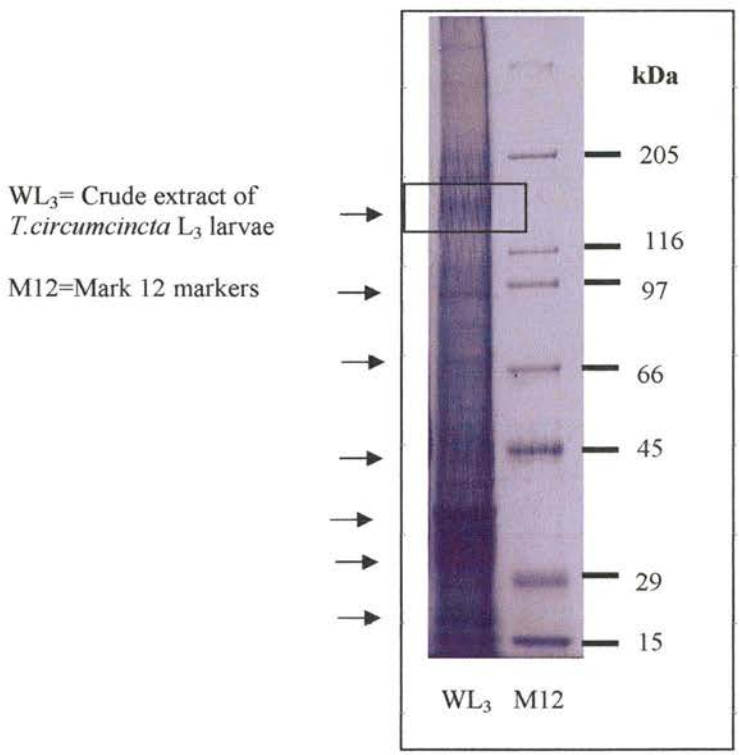
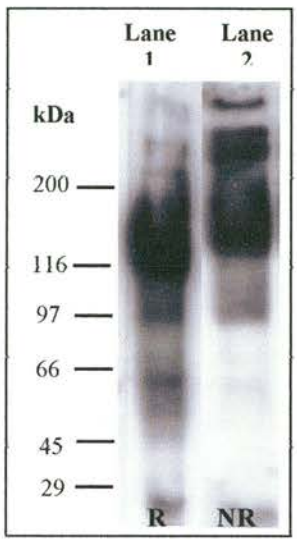


Figure 3.2 Colloidal Coomassie staining of SDS-PAGE gel to determine protein bands present within whole *T.circumcincta* L_3 antigen

The IgE binding components of whole *T.circumcincta* larval antigen were subsequently determined using western blot analysis. Figure 3.3 shows that under reduced conditions there was a major IgE binding component at approximately (~) 120kDa but under non-reduced conditions the major IgE binding component existed at ~150kDa, although two higher molecular weight bands were also observed in some blots. However, these two higher bands were not always present when probed

with different sera. The immunodominant band present at ~150kDa in lane 2 was consistently present and had the same molecular weight as one of the main protein bands present in the colloidal gel in figure 3.2 (see black box). From these SDS-PAGE studies it was determined that the whole *T.circumcincta* L_3 antigen of interest had a molecular weight of ~150kDa under non-reduced conditions.



Lane 1 = Under Reduced conditions

Lane 2 = Under Non-reduced conditions

Figure 3.3 Western blot analyses to show the IgE binding components within whole *T.circumcincta* L_3 extract

3.3.2 Purification of *T.circumcincta* whole larval antigen by gel permeation chromatography

Figure 3.4 displays the resulting HPLC trace from the S300 purification of *T.circumcincta* whole soluble larval antigen. Each of the 1ml fractions collected was tested for IgE by dot blot analysis to confirm the presence of the immunodominant larval surface antigen. The IgE binding immunodominant larval surface antigen as determined in 3.3.1 was shown to be eluted from the S300 column in fractions 21 to 44 as indicated by the dotted lines on the trace.

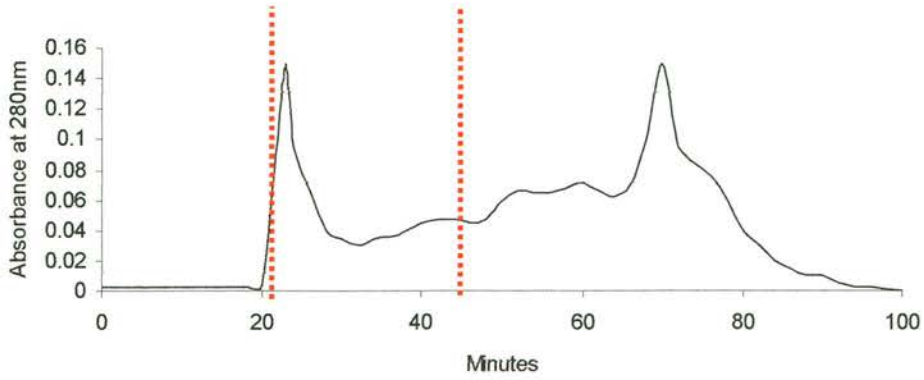


Figure 3.4 The trace of S300 purification of *T.circumcincta* whole soluble larval antigen

3.3.3 Purification of the immunodominant *T.circumcincta* larval antigen by affinity chromatography.

Although affinity purification resulted in a comparatively pure preparation of allergen, as demonstrated by a faint single band on Coomassie blue stained gel (unpublished observations), the yields were very low when compared to the gel filtration purified product. Furthermore, the immunoaffinity purified product reacted only weakly on western blots, compared to the S300 fraction (figure 3.5), which was therefore employed in all the subsequent studies.

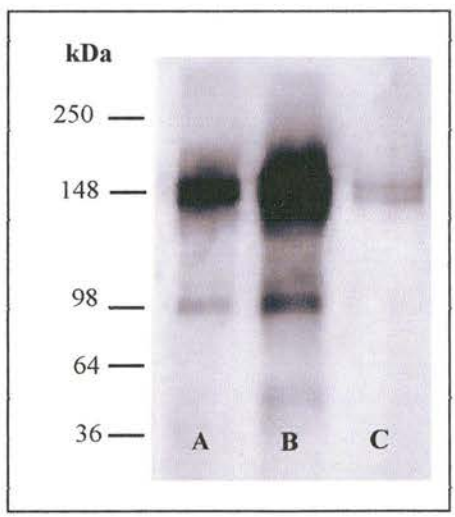


Figure 3.5 Western blot analysis to show IgE reactivity in preparations of *T.circumcincta* L3 allergen purified by gel filtration or affinity chromatography. Lane (A) S300 fraction, (B) Whole larval antigen, and (C) Affinity chromatography

3.3.4 Characterisation of S300 purified *T.circumcincta* L_3 antigen

Figure 3.6 shows the protein bands present within the whole *T.circumcincta* L_3 antigen and the S300 purified *T.circumcincta* L_3 antigen. The gel showed that the S300 purified antigen extract displayed a significant protein band at approximately 150kDa and also at 45kDa. The whole soluble larval extract also displayed these two proteins plus other protein bands.

WL_3= whole larval *T.circumcincta* antigen

S300= S300 purified *T.circumcincta* antigen

M12= Mark 12 markers

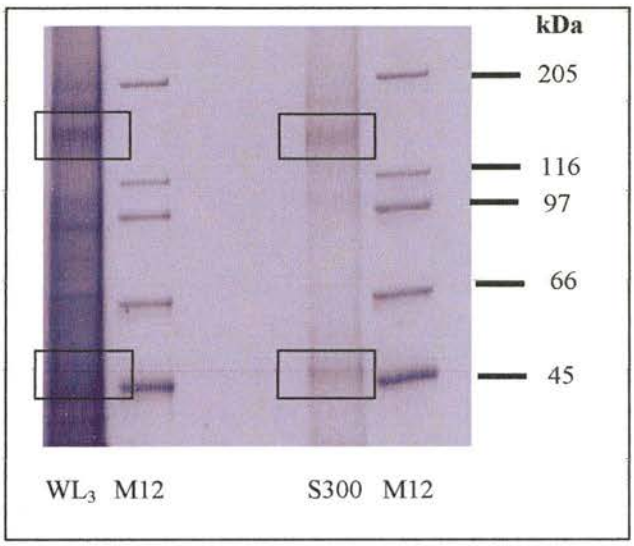
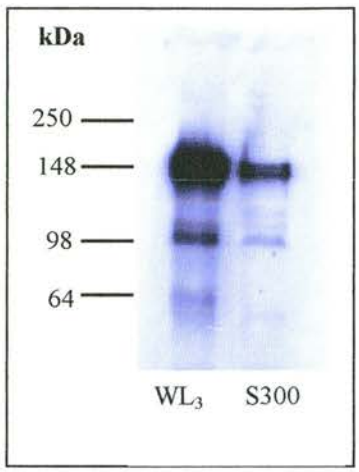


Figure 3.6 Colloidal Coomassie staining of SDS-PAGE gel to determine protein bands present within whole and S300 purified *T.circumcincta* L_3 antigen

Comparisons were then made with the IgE binding components between the crude and S300 purified L_3 *T.circumcincta* antigens. Figure 3.7 demonstrated that a major band appeared at approximately 150kDa in both extracts, and a minor band at 98kDa was also observed. A minor 64kDa reactive band in the whole extract was absent in the S300 fraction.



WL_3 = Whole larval *T.circumcincta* antigen

S300 = S300 purified *T.circumcincta* antigen

Figure 3.7 Western blot analysis to show the IgE binding proteins within both whole *T.circumcincta* L_3 antigen and S300 purified *T.circumcincta* L_3 antigen

3.3.5 Periodate treatment of S300 purified L_3 *T.circumcincta* antigen

The average OD results from the periodate treated ELISA method described in 3.2.8 are shown in table 3.3. The results demonstrated that the OD's of the periodate treated S300 purified *T.circumcincta* L_3 antigen reduced by approximately 20%.

ELISA Plate Column	Average OD @ 492nm
1 (Neg)	0.000
2 (Neg)	0.000
3 (Pos)	1.220
4 (Pos)	1.148
5 (5mM)	0.859
6 (10mM)	0.849
7 (15mM)	0.826
8 (20mM)	0.806
9 (50mM)	0.799
12GH (Pos)	0.962

Table 3.3 Average OD results of periodate treated ELISA

3.3.6 Glycoprotein analysis of S300 purified *T.circumcincta* L_3 antigen

The results of the glycoprotein staining carried out using two different methods are displayed in figure 3.8 and 3.9. Figure 3.8 demonstrated the presence of a coloured band at ~150kDa and ~120kDa in the non-reduced and reduced sample of S300 purified *T.circumcicnta* L_3 antigen, respectively. This result established that the high molecular weight immunodominant larval antigen was a glycoprotein. Figure 3.9 confirmed this result showing a fluorescent band at ~150kDa and a much fainter band at ~120kDa in the non-reduced and reduced *T.circumcincta* larval antigen sample, respectively. The Emerald 300 Glycoprotein Gel Stain Kit appeared to be more sensitive than the Gelcode® Glycoprotein staining kit due to the presence of more glycoprotein bands.

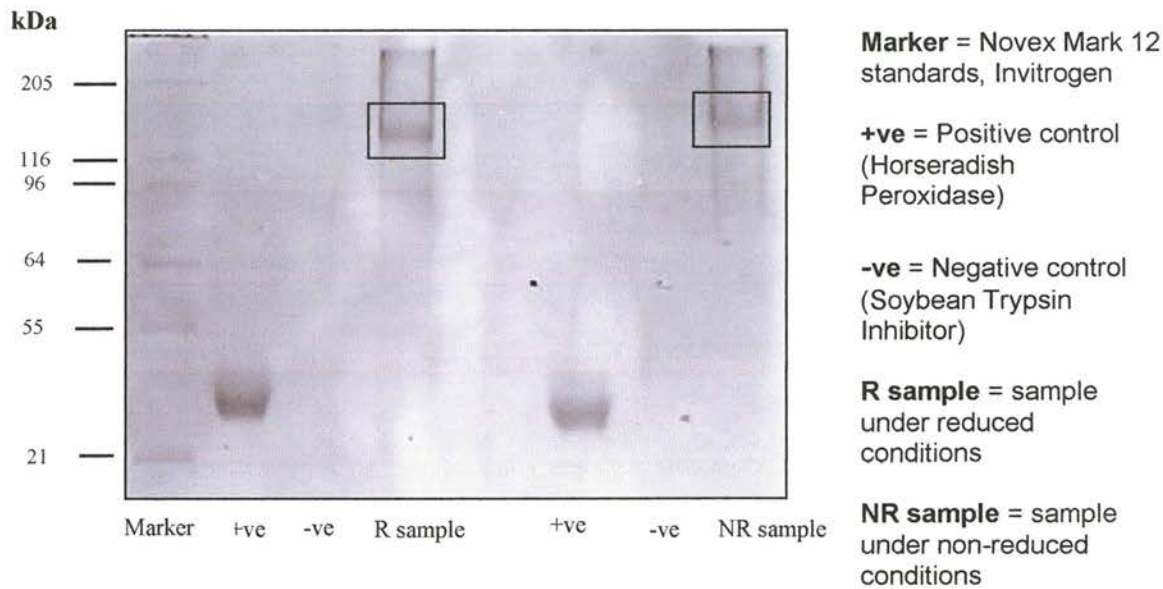


Figure 3.8 Glycoprotein detection using Gelcode® Glycoprotein Staining Kit (PIERCE)

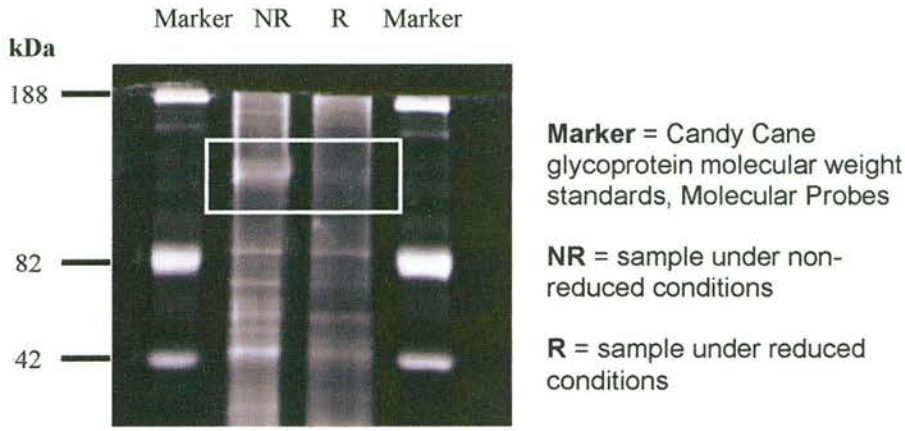


Figure 3.9 Glycoprotein detection using Pro-Q™ Emerald 300 Glycoprotein Gel Stain Kit (P-21855)(Molecular Probes)

3.3.7 Lipoprotein analysis of S300 purified *T.circumcincta* L_3 antigen

The result of the lipoprotein staining is shown in figure 3.10. Lipoproteins were clearly shown in the positive control but no staining was observed in the lane containing the S300 purified *T.circumcincta* L_3 antigen.

Figure 3.10 Lipoprotein detection using Nile Red (SIGMA)

3.3.8 Mass Spectrometry - Tryptic Peptide Mapping from SDS-PAGE gels

Figure 3.11 demonstrates the mass spectra obtained from the S300 purified *T.circumcincta* L_3 antigen sample

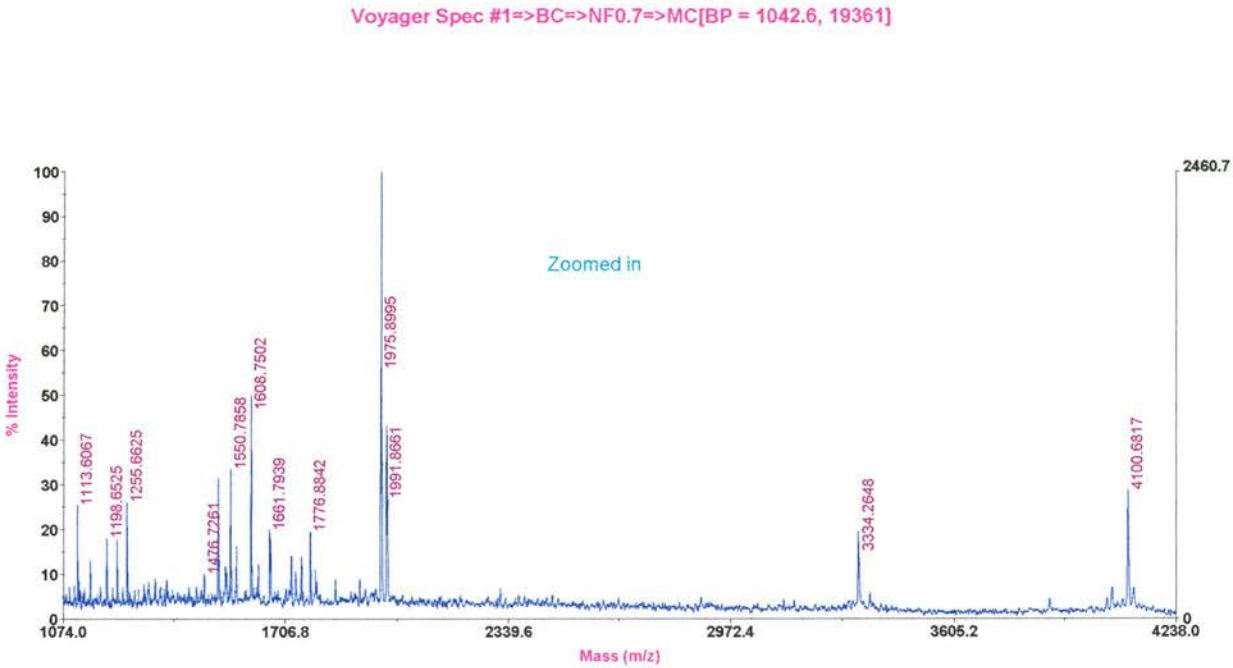


Figure 3.11 Mass spectra of S300 T.circumcincta L_3 antigen

No significant matches using the peptide masses of this sample were found with other known proteins using the MS-FIT software.

3.3.9 N-Terminal amino acid sequence report of S300 purified *T.circumcincta* L_3 antigen

Table 3.4 displays the N-terminal amino acid sequence report of S300 purified *T.circumcincta* L_3 antigen. During the sequencing of the sample, the signal was noisy and so it became impossible to generate sequence data after 12 cycles. Sequence data for the first 12 residues was however obtainable. The second and third row of table 3.3 displays possible alternatives of the amino acids.

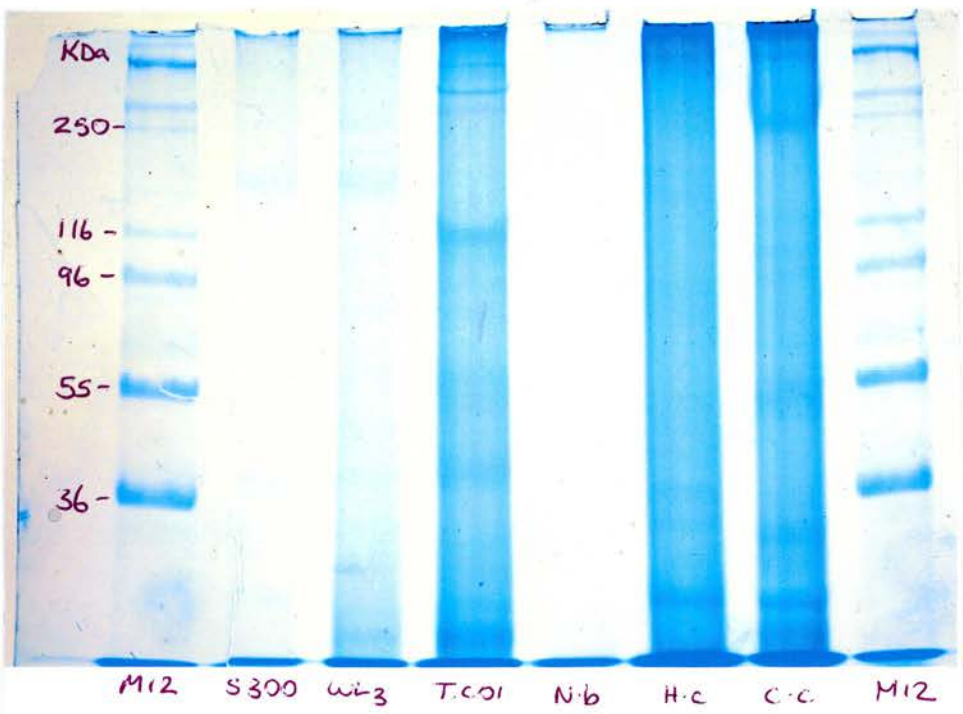
Cycle	1	2	3	4	5	6	7	8	9	10	11	12
	*K	T	V	G	Q	E	T	A	P	N?	R	H
	D?	N	L	A		W	R	N	D	X		D
	X	A	A			G	S					

Table 3.4 N-terminal amino acid sequence report of S300 purified T.circumcincta L_3 antigen

* = K in cycle 1 is often seen with PVDF samples so may be an artifact.

3.3.10 Characterisation of whole L_3 larval antigen from six different parasite species

Using the Colloidal Coomassie stain method in 3.2.12, it was possible to visualise the protein bands present within whole L_3 antigen of four different parasite species (prepared in 2.7.1). The resulting gel is displayed in figure 3.12. Protein bands were very faint, but it can be seen that the other species of parasite did not share this high molecular weight protein at ~150kDa.



M12 = Novex Mark 12 standards (Invitrogen)

C.c = Whole larval extract of *C.curticei*

H.c = Whole larval extract of *H.contortus*

N.b = Whole larval extract of *N.battus*

T.col = Whole larval extraxt of *T.colubriformis*

WL_3 = Whole larval extract of *T.circumcincta*

S300 = S300 purified larval extract of *T.circumcincta*

Figure 3.12 Colloidal Coomassie staining of SDS-PAGE gel to determine protein bands present within the whole L_3 antigen of six different species of sheep parasite (→ = 150kDa protein)

The IgE binding components of these four different larval antigens were subsequently determined using western blot analysis (see figure 3.13). The blot demonstrated that both the S300 purified *T.circumcincta* antigen and the whole larval *T.circumcincta* antigen were the only extracts that displayed an IgE reactive band at ~150kDa. The *T.colubriformis* larval extract showed IgE cross-reactive protein bands at ~120kDa and also at ~ 45kDa. The *N.battus* larval extract displayed no IgE reactivity and the *H.contortus* and *C.curticei* larval extracts showed some IgE cross-reactivity at a molecular weight of ~50kDa.

See figure 3.12 for definition of abbreviations labelled on the blot

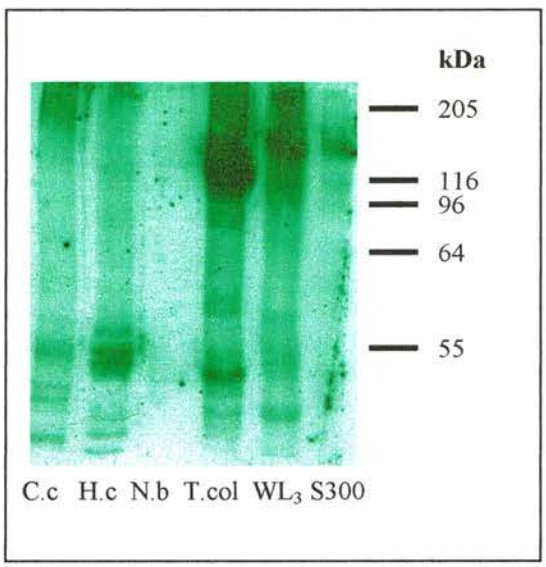


Figure 3.13 Western blot analysis to show the IgE binding components within whole L_3 extract from six different sheep parasites

3.3.11 Comparisons between infective L_3, L_4 and L_5 *T.circumcincta* whole larval antigens

The protein bands present within the L_3, L_4, and L_5 *T.circumcincta* whole larval antigens are shown in figure 3.14. It was shown that the L_3 and L_4 *T.circumcincta* antigens displayed the high molecular weight protein at ~150kDa. However, this protein band was not clearly distinguished in the L_5 *T.circumcincta* antigen.

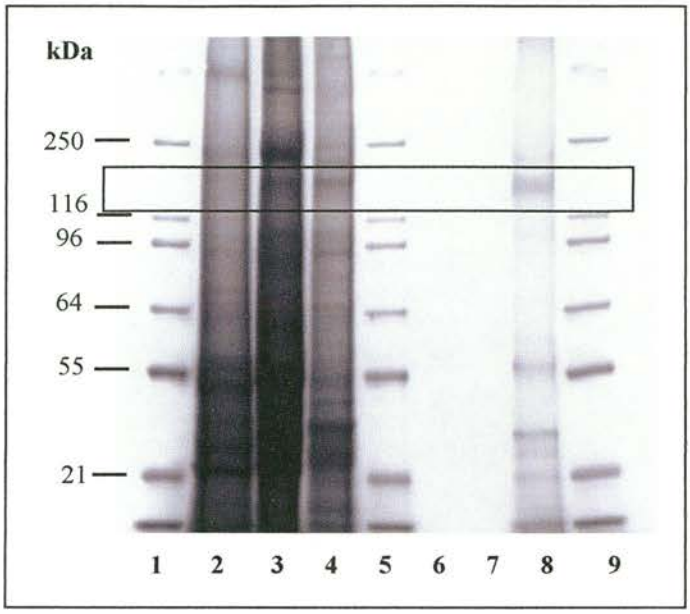


Figure 3.14 Colloidal Coomassie staining of SDS-PAGE gel to determine protein bands present within L_3, L_4 and L_5 *T.circumcincta* whole larval antigens

The IgE binding components of the L_3, L_4, and L_5 *T.circumcincta* whole larval antigens are shown in figure 3.15. A dominant IgE binding protein at approximately 150kDa was demonstrated in the crude and S300 purified L_3 preparation. However, in the whole L_4 and L_5 *T.circumcincta* extracts little or no IgE reactivity to this protein was observed. A very faint IgE binding component was, however, visible at approximately 110kDa in the whole L_4 extract.

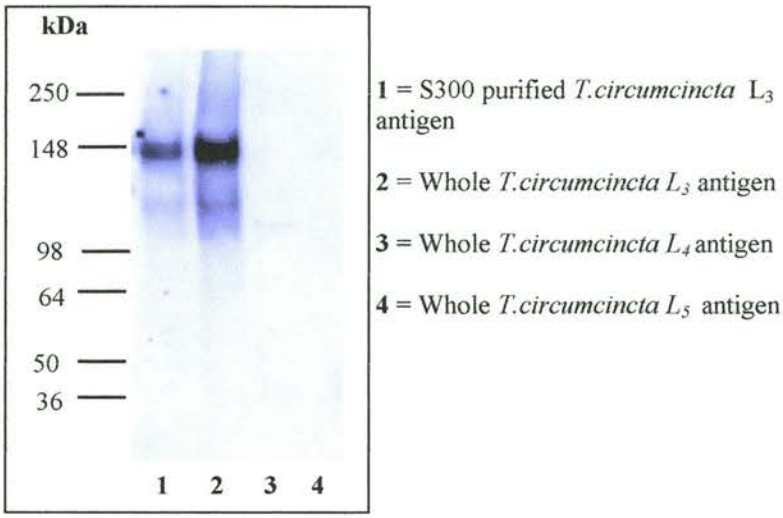


Figure 3.15 Western blot analysis to show the IgE binding components within L_3, L_4, and L_5 T.circumcincta whole larval antigens

3.3.12 Western blot analysis to determine IgA and IgG binding proteins of S300 *T.circumcincta* L_3 antigen

Western blot analysis was carried out to determine the IgA and IgG binding properties of the S300 purified *T.circumcincta* L_3 antigen. Figure 3.16 demonstrates that the high molecular weight immunodominant *T.circumcincta* L_3 antigen was recognised strongly with IgG antibodies, and some reaction with IgA antibody was also observed.

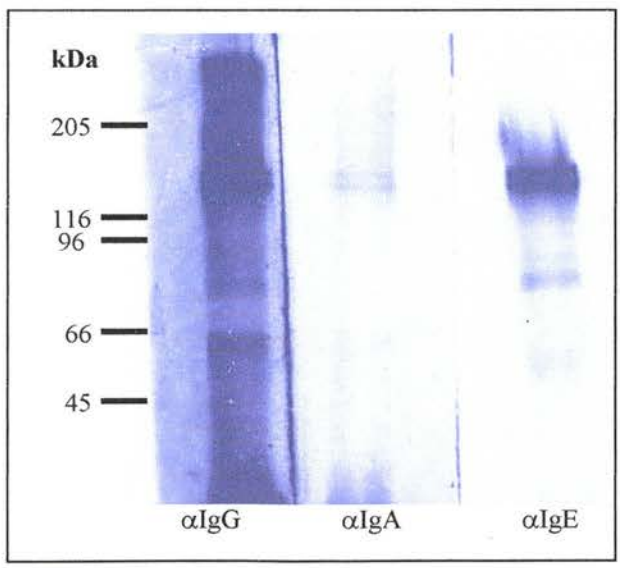


Figure 3.16 Western blot analysis to determine IgA and IgG binding properties of S300 purified *T.circumcincta* L_3 antigen. Reactivity with αIgE is shown for comparison

3.3.13 Development of parasite specific IgE ELISA

The graph shown in figure 3.17 demonstrates the parasite specific IgE antibody in the sera of ten lambs at a specific time point in the grazing season. The first column shows ODs following assay of the whole sera, and the second column represents the ODs obtained from the same sera after extracting the IgE. Following IgE extraction, generally higher levels of IgE antibody were detected in these samples.

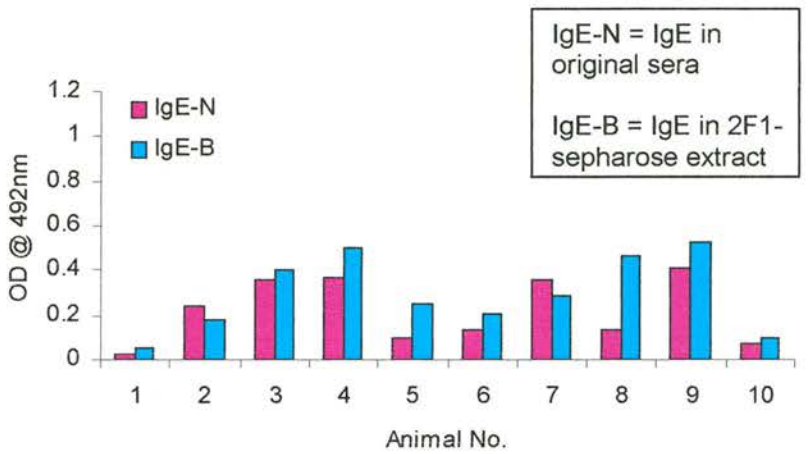


Figure 3.17 Parasite specific IgE levels of 10 lambs using the standard ELISA on whole sera, and following IgE extraction.

The IgE bead-eluted samples were also assayed for the presence of IgG and IgA antibodies by ELISA. Surprisingly, about 30% of the samples indicated high levels of contaminating IgG (figure 3.18) and all lambs displayed trace levels of IgA antibody (figure 3.19).

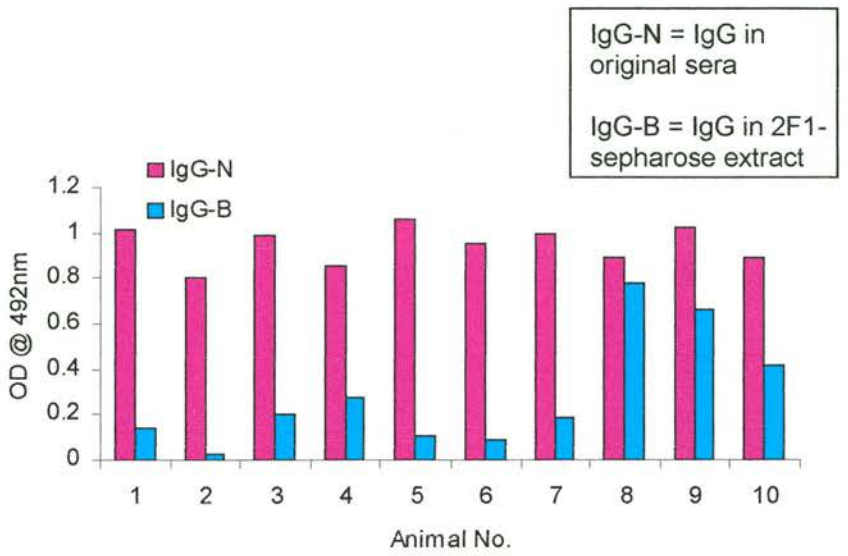


Figure 3.18 Parasite specific IgG levels of 10 lambs using the standard ELISA on whole sera, and following IgE extraction

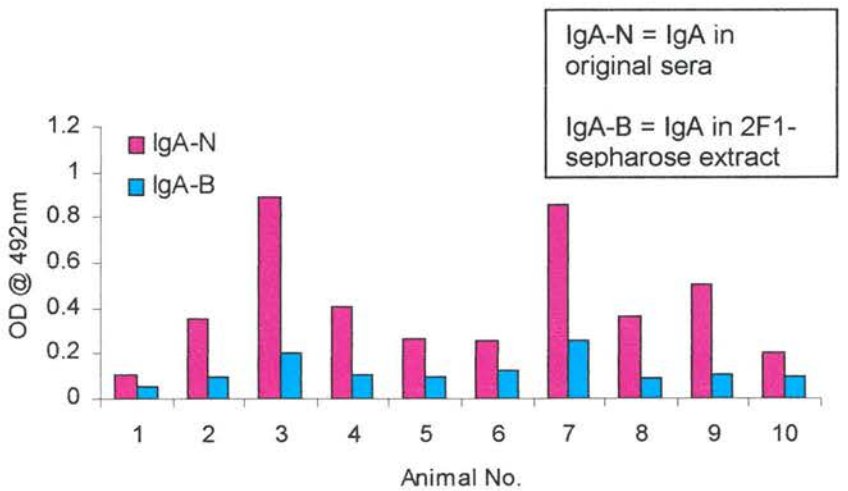


Figure 3.19 Parasite specific IgA levels of 10 lambs using the standard ELISA on whole sera, and following IgE extraction.

3.4 DISCUSSION

The initial aim of this study was to isolate and purify the antigen, which has been shown to be located predominantly on the surface of *Teladorsagia circumcincta* L_3 larvae and which specifically binds to ovine IgE (Huntley *et al.*, 2001).

An S300 gel filtration column was chosen to fractionate the soluble extract because the size of the immunodominant surface allergen had been previously shown to be approximately 150 kDa, and this is the optimum medium for fractionating biomolecules of that size. Affinity chromatography was employed in an attempt to purify the antigen further and also increase recovery. Figure 3.5 shows that further purification has been achieved by affinity chromatography but the relative recovery of protein was so low that it was decided not to pursue this method. The reason for this poor recovery is unclear, but it is possible that the rabbit antibody was of insufficient quality or quantity. Preliminary studies employed to establish the presence of glycoproteins within the high molecular weight antigen confirmed that glycoproteins were present within the composition of the S300 purified *T.circumcincta* L_3 antigen. Previous work has provided evidence that carbohydrate residues are associated with the "allergenicity" of some Schistosome proteins (Cutts & Wilson, 1997). Recent investigations have also reported that IgE antibodies from serum of Schistosoma mansoni infected mice and *H.contortus* infected sheep recognise complex-type N-glycans (van Die *et al.*, 1999). These glycoproteins consist of core $\alpha 1 \rightarrow 3$-Fucosylated and $\beta 1 \rightarrow 2$-Xylosylated N-glycans. It was suggested that the core $\alpha 1 \rightarrow 3$-Fucosylated and/or the $\beta 1 \rightarrow 2$-Xylosylated residues may be epitopes for both IgE from *Schistosoma mansoni* infected mice and *H.contortus* infected sheep. However, studies have since shown that the sheep nematode *H.contortus* contains glycoproteins consisting of $\alpha 1 \rightarrow 3$-Fucosylated residues but not $\beta 1 \rightarrow 2$-Xylosylated residues (van Die *et al*, 1999). This study therefore suggests that the core $\alpha 1 \rightarrow 3$-Fucosylated N-glycan was the epitope that reacted with IgE from *H.contortus* infected sheep. Van Die *et al.* (1999) have also demonstrated that many helminths from different orders of the phyla Platyhelminths and Nematodes produce glycoproteins containing core $\alpha 1 \rightarrow 3$-Fucosylated N-glycans. The suggestion is made that the core $\alpha 1 \rightarrow 3$-Fucosylated N-glycan may contribute to the induction of a Th2 response leading to the production of IgE.

Although this N-glycan is a common trait of several antigens it is not known whether identical or similar glycoproteins are involved in the composition of the high molecular weight S300 *T.circumcincta* L_3 antigen. However, in the present study it is unlikely that glycoproteins were a major component of the IgE reactive epitopes, since only a modest (20%) reduction was observed following periodate treatment.

This initial chapter has also demonstrated that there are no lipids present within the structure of S300 purified *T.circumcincta* L_3 antigen.

Mass spectrometry analysis using the MALDI-TOF was employed to obtain mass spectra of the S300 purified *T.circumcincta* L_3 antigen. The peptide mass information was used to determine if there were any matches with other proteins found on the MS FIT database. No significant matches were observed, possibly due to the protein of interest originating from a species where the genome is not fully sequenced. Additionally, postranslational modifications such as glycosylation can cause problems when trying to identify proteins. These problems can be overcome by the use of the Q-Trap LC/MS/MS system (Applied Biosystems). This instrument has different scan modes to enable derivation of maximum sequence information from peptides and can improve the signal to noise ratio by eliminating singly charged ions from the spectra. Additionally, it can identify sites of postranslational modifications such as glycosylation.

N-terminal amino acid analysis using the Procise 494 Protein Sequencer (Applied Biosystems) was used to acquire an amino acid sequence report of the S300 purified *T.circumcincta* L_3 antigen. The report consisted of 12 amino acids although no significant matches with other proteins could be found using the amino acid database search. Explanations for not obtaining a protein match could be due to sample purity, insufficient material and/or interfering substances.

It was demonstrated that the high molecular weight, 150kDa protein present within a sample of S300 purified *T.circumincta* L_3 antigen was not identified in the crude L_3 extracts of *T.colubriformis*, *N.battus*, *H.contortus* and *C.curticei*. The IgE binding components of these four parasite species were also determined and demonstrated that all the parasite extracts except *N.battus* showed some IgE cross-reactivity but none of the parasites possessed the 150kDa allergen present in the *T.circumcinta* L_3 antigen.

Studies were carried out to investigate the crude extracts of third, fourth and fifth stage *T.circumcincta* larval antigens in relation to their IgE reactivity. It was found that the fourth larval extract did not possess the high molecular weight protein band that was present on the third stage larval extract but did show a very faint IgE binding component at a molecular weight of approximately 110kDa. The fifth stage larval extract showed no IgE reactivity. These results correspond to previous investigations undertaken by Huntley *et al.* (1998a) suggesting that IgE responses to *T.circumcincta* are predominantly to the L_3 infective larvae and not to the L_5 larvae. It is well known that parasites generate stage-specific antigens during their life cycles (Jungery *et al.*, 1982; McKeand *et al.*, 1996; Darwish *et al.*, 1996), and some of these may be dominant allergens which elicit strong IgE antibody responses. Examples of the latter are mentioned previously in the introduction including allergens such as *Ascaris spp.* (Muto *et al.*, 2001), *Brugia malayi* (Paxton *et al.*, 1993) and *schistosomula* (Santiago *et al.*, 1998), and this study shows that *T.circumcincta* generates a dominant L_3 allergen that is involved in stimulating IgE antibody responses. These results suggest that parasite development may have a profound influence on the type of antibodies being produced which may be relevant in the expression of immunity generated and will be discussed in more detail in chapter four.

Parasite specific IgE ELISAs in sheep have been employed in various studies since the development of a mouse anti-ovine IgE monoclonal (Windon *et al*, Veterinary Immunology and Immunopathology, in press; Shaw *et al.*, 1997 Kooyman *et al.*, 1997) and more information has thus become available in understanding the IgE response to nematode infections in ruminants. It was reported in this study that, in addition to the S300 *T.circumcincta* L_3 antigen recognising IgE antibodies, IgA and IgG antibodies were also identified by this antigen. This suggested that IgA and IgG antibodies could be competing for the same epitope on the antigen as the IgE antibodies and so an attempt was made to develop an ELISA, without the presence of competing IgG and IgA antibodies. This was achieved by extracting IgE from sera prior to being assayed by ELISA. The results did show an increase in IgE antibody level when the IgE was extracted from the sera, although the differences were marginal and did not alter the overall pattern or magnitude of the

results from individual sera. In view of the practical difficulties in extracting IgE from large numbers of samples, all subsequent assays were performed on whole sera. The results also showed that the IgA levels were present in negligible amounts after the sera had been IgE purified. However, 30% of the animals still showed high levels of IgG antibody after the sera had been treated. The monoclonal antibody used to pull out the IgE from the sera is highly specific to IgE (Windon *et al,* Veterinary Immunology and Immunopathology, in press) and hence, there should be no IgG antibodies present in the sera. One possibility however, is that IgE and IgG antibodies may be forming a complex, since IgE-IgG complexes have been found in other species. For example; Boluda *et al.*, 1995 have reported that immune stimulation in atopic human individuals is not restricted to the IgE isotype, but equally affects the IgG-producing antibody systems. The relationship observed may either be due to common pathways in the production of both antibody classes in atopic people, or may be explained by preferential binding of allergens to circulating IgE-IgG immune complexes. Sato *et al.* (1998) have also described IgE-IgG complexes found in sera samples of mice.

Having successfully developed the assay for the detection of IgE anti-high molecular weight *T.circumcincta* allergen, experiments were now performed to monitor the generation of these antibodies in lambs during their first grazing season on a pasture contaminated with *T.circumcincta.*

Chapter 4

Antibodies as Markers for Responsiveness against *Teladorsagia* Infections in Greyface Suffolk Cross Lambs

4.1 INTRODUCTION

Increasing levels of drug resistance and the desire to minimise chemical usage in food animals are two pressing reasons for developing novel approaches to the control of economically important parasitic nematodes in livestock. Selective breeding of animals that are more able to mount protective immunity to infection is one attractive option because of its capacity to reduce reliance upon chemotherapy (Beh & Maddox, 1996). The sensitivity of selection methods to identify resistant animals using FECs as a phenotypic marker would be improved by the inclusion of additional phenotypic markers, particularly where these were simple and relatively cheap to use. A simple serological assay, such as an ELISA, could provide a rapid, sensitive and relatively inexpensive quantitative measure of responsiveness against gastrointestinal nematodes and would therefore be an ideal tool for on farm selection programmes.

Previous work at the Moredun Research Institute has already provided preliminary evidence that circulating IgE might provide a viable phenotypic indicator of individual resistance (Huntley *et al.*, 1998a & b). The overall aim of this investigation was to examine a variety of immunological markers, which are associated with resistance and susceptibility, principally focussing on the significance of peripheral serum IgE and IgA as markers of immune response against gastrointestinal nematodes in sheep. Differences in both the rate and extent to which individuals respond against gastrointestinal nematodes have been recognised for many years (Barger, 1987) and form the basis for genetic selection programmes in sheep (Albers *et al,* 1987; Windon 1990; Woolaston *et al.,* 1990). Previous studies have also highlighted the value of using identified responders and non-responder animals as a means of examining mechanisms regulating gastrointestinal nematodes (Patterson *et al*, 1996a, 1996b). The previous chapter indicated that IgE might play a role in a number of nematode infections. The fact that specific IgE antibody levels in sheep increase during a natural infection of *T. circumcincta* with the IgE response being largely directed against the infective larval stages led to the identification and production of L_3 *T.circumcincta* antigen associated with the response. The aim of the current studies was to use this antigen in an ELISA in a pilot study to determine the concentrations of both parasite specific IgE and IgA antibodies in naturally

infected lamb sera. These data were used in conjunction with individual FECs in a preliminary examination of the relationship between antibody level and FEC. Previous investigations have observed the relationship between circulating antibodies and FEC in sheep. Huntley *et al.*, 2001 demonstrated that Dorset Finn lambs had significantly higher peripheral IgE antibody responses when cumulative FECs were low. Additionally, Strain *et al.* (2002) reported that Scottish Blackface lambs with an increased peripheral IgA activity had lower FECs.

4.2 MATERIALS AND METHODS

Serum samples used in this pilot study were acquired from a previous experiment that was carried out at the Moredun in 1995. Lambs were bled fortnightly and serum was stored at –20°C for subsequent analysis (see 2.3).

4.2.1 Animals

Details of the Greyface X Suffolk lambs used in this study are described in 2.1. 80 Greyface X Suffolk lambs were put out on to pasture naturally infected predominantly with *T.circumcincta* in mid to late April. Anthelmintic treatment was given to the animals initially in mid to late May (May 18th 1995) to control *N.battus* and a summer drench (July12th 1995) to control other gastrointestinal nematodes. Broad-spectrum anthelmintics were used under the direction of the manufacturer's instructions.

4.2.2 Parasitological Parameters

Lambs were faecal sampled *per rectum* fortnightly. Faecal consistencies were monitored as described in 2.2.2. FECs were determined using a flotation method described in 2.2.3. Lambs were ranked in order of their cumulative resistance to nematode infection as determined by their individual FECs for the middle of the season (1st June–12th July 1995) and also the end of the season (8th August-6th September 1995). The FECs were ranked because the distribution of egg counts were very skewed. The ten lambs that had the lowest overall egg count were termed "responders" and the animals with the highest egg count were termed "non-responders". The average of the FEC rankings for each of these time periods was

then calculated and an overall rank was given for each of the animals. The middle and end season time points were chosen by taking the two periods after the anthelmintic treatment dates, 18th May and 12th July.

4.2.3 Parasite specific ELISAs using S300 purified *T.circumcincta* L_3 antigen

Parasite specific IgE and IgA ELISAs were carried out using the serum collected from the lambs over the grazing season (see 2.4.2). ELISA plates were coated with S300 purified *T.circumcincta* L_3 antigen at 1μg/ml. For the parasite specific IgE ELISAs the secondary antibody used was anti-IgE 2F1 (Windon *et al*, in press) at 1/1000 dilution. For the parasite specific IgA ELISAs the secondary antibody used was anti-IgA (Serotec, Oxford, UK) at 1/2000 dilution.

4.2.4 Statistical analysis

Statistical analysis was employed as described in 2.7. Results were expressed as group means and SEMs. Differences between groups of lambs were compared using a two-tailed Student's t-test. Correlations between two variables were calculated using Spearman's rank correlation to see whether the two variables to be tested covary. $P<0.05$ was considered statistically significant in both statistical tests.

4.3 RESULTS

4.3.1 Selected responder and non-responder lambs

Table 4.1 displays the animal numbers that were selected as responders or non-responders using their FEC rankings from three different time periods across the grazing season (middle, end and overall). The responder and non-responder lambs chosen using the middle season FEC rankings were not the same as the lambs selected using the end of season FEC rankings. There was only one non-responder that was observed in both time periods and that was animal number 1428. Additionally, animal number 1567 was a responder during the middle of the season but became a non-responder towards the end. The overall selected responder lambs included 5 responder and 7 non-responder animals selected from the middle and end season all together.

Middle Season		End Season		Overall Season	
R	NR	R	NR	R	NR
1535	1428	1485	1428	1485	1428
1545	1544	1525	1484	1488	1501
1517	1375	1386	1465	1525	1544
1519	1387	1490	1398	1535	1375
1531	1436	1498	1421	1498	1436
1539	1360	1358	1449	1798	1466
1553	1415	1422	1459	1510	1415
1554	1441	1456	1470	1518	1421
1566	1442	1460	1517	1519	1442
1567	1456	1509	1567	1554	1455

Table 4.1 Responders and Non-responders selected using middle, end and overall season F.E.C rankings

4.3.2 Lamb faecal egg counts

Figure 4.1 shows the FECs for the responder and non-responder lambs from the two different time intervals (middle and end) and overall over the grazing season. The average FEC of the flock was included on each graph. The mid season graph (figure 4.1) showed that the responders selected for this period of time had relatively low FECs throughout the season. The non-responders FECs, however, increased to approximately (~) 700 eggs per gram of faeces (epg) in late June displaying a noticeable difference in egg production between the two groups of lambs at this point. The FECs of the non-responder lambs subsequently decreased and followed a similar pattern to that of the responders for the remainder of the season. The end season graph (figure 4.1) demonstrated that both groups of lambs showed similar within-season patterns of egg count and no noticeable differences were observed until towards the end of the season where a slight divergence was seen between the responder and non-responder lambs. The overall season graph (figure 4.1) showed that the two groups of lambs followed the same initial pattern, but the responders and non-responders diverged by late June with the latter group reaching egg counts at

~600 epg and the egg counts of the responders remaining low for the remainder of the season.

The pasture that the Greyface x Suffolk lambs grazed on was mainly contaminated with *T.circumcincta*. The flock of lambs produced on average 2143 million eggs across the grazing season. Table 4.2 contains details of the estimated % pasture contamination that the responders and non-responders across the grazing season were responsible for.

	% Pasture contamination	
	Responders	**Non-Responders**
Mid Season	8	21
End Season	11	10
Overall Season	4	23

Table 4.2 Percentage Pasture contamination of responder and non-responder lambs over the grazing season

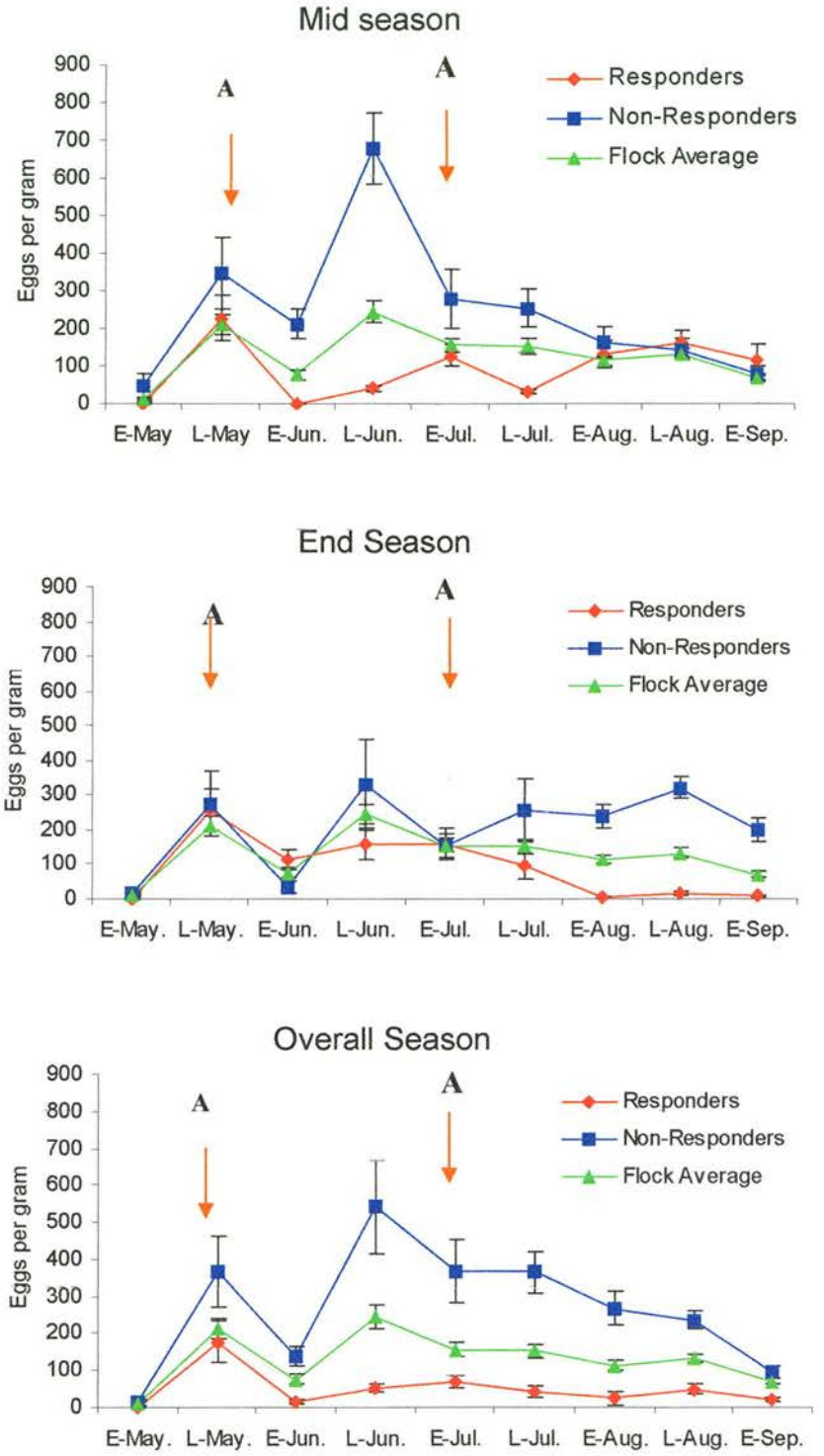


Figure 4.1 Average trichostrongylid faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season.

A = anthelmintic treatment

4.3.3 Parasite Specific ELISAs

4.3.3.1 IgE Antibody ELISAs

Figure 4.2 shows the parasite specific IgE antibody levels for the responder and non-responder lambs from the three different time points (middle, end and overall) over the grazing season. All three graphs in figure 4.2 followed the same initial pattern up to late July. They displayed a very gradual increase in IgE antibody levels at the beginning of the season and then towards the end IgE antibody levels increased approximately two fold. Non-responder and average groups, however, displayed a sudden decrease in IgE antibody concentration in late August but increased again in early September.

The mid season graph showed a significant difference between responders and non-responders over the last three time points ($p<0.05$) with the responders displaying a greater increase in IgE antibody levels. The end season graph demonstrated no significant differences in IgE antibody titre between the two groups of lambs. The overall season graph showed a significant divergence between the responders and non-responders towards the end of the season (E-Aug.- E-Sep) ($p<0.05$) with the former group of lambs demonstrating a higher IgE antibody concentration. A student's t-test was employed to carry out these comparisons.

4.3.3.2 IgA Antibody ELISAs

Figure 4.3 shows the parasite specific IgA antibody levels for the responder and non-responder lambs from the three different time points (middle, end and overall) over the grazing season. A general pattern was seen in the three graphs in figure 4.3. All IgA antibody titres were higher at the end of the season compared to those detected at the beginning of the season in both groups of lambs for each graph. The mid season and overall season graph showed no significant differences in IgA antibody concentrations between the two groups of lambs over the last three time points of the grazing season. The overall season graph showed a sudden decrease in IgA antibody levels in the responder group in early August. A divergence between the two groups of lambs in IgA antibody titre in the end season graph was observed from late July onwards although this difference was not significant.

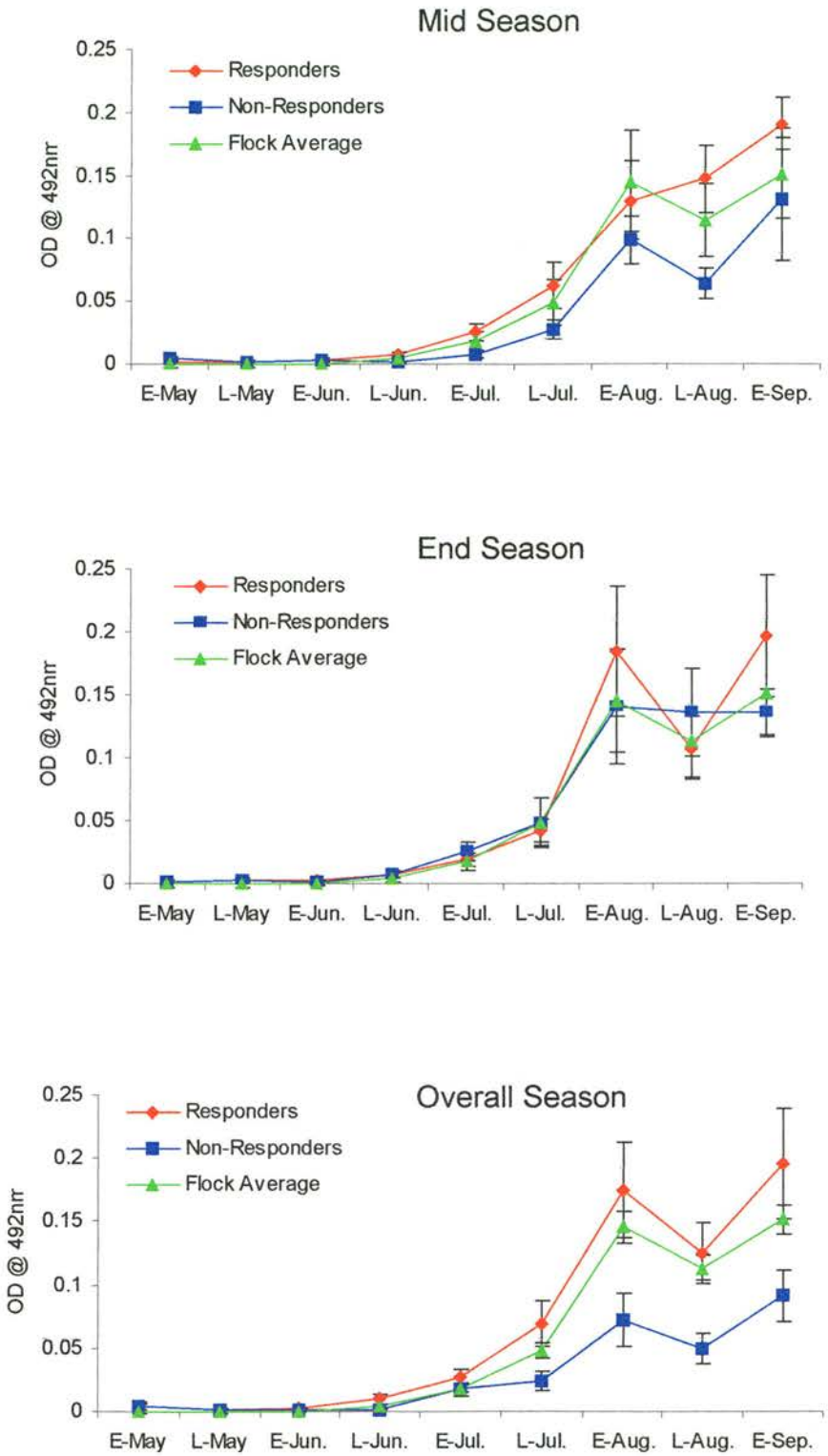


Figure 4.2 Average Parasite specific IgE Antibody levels (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

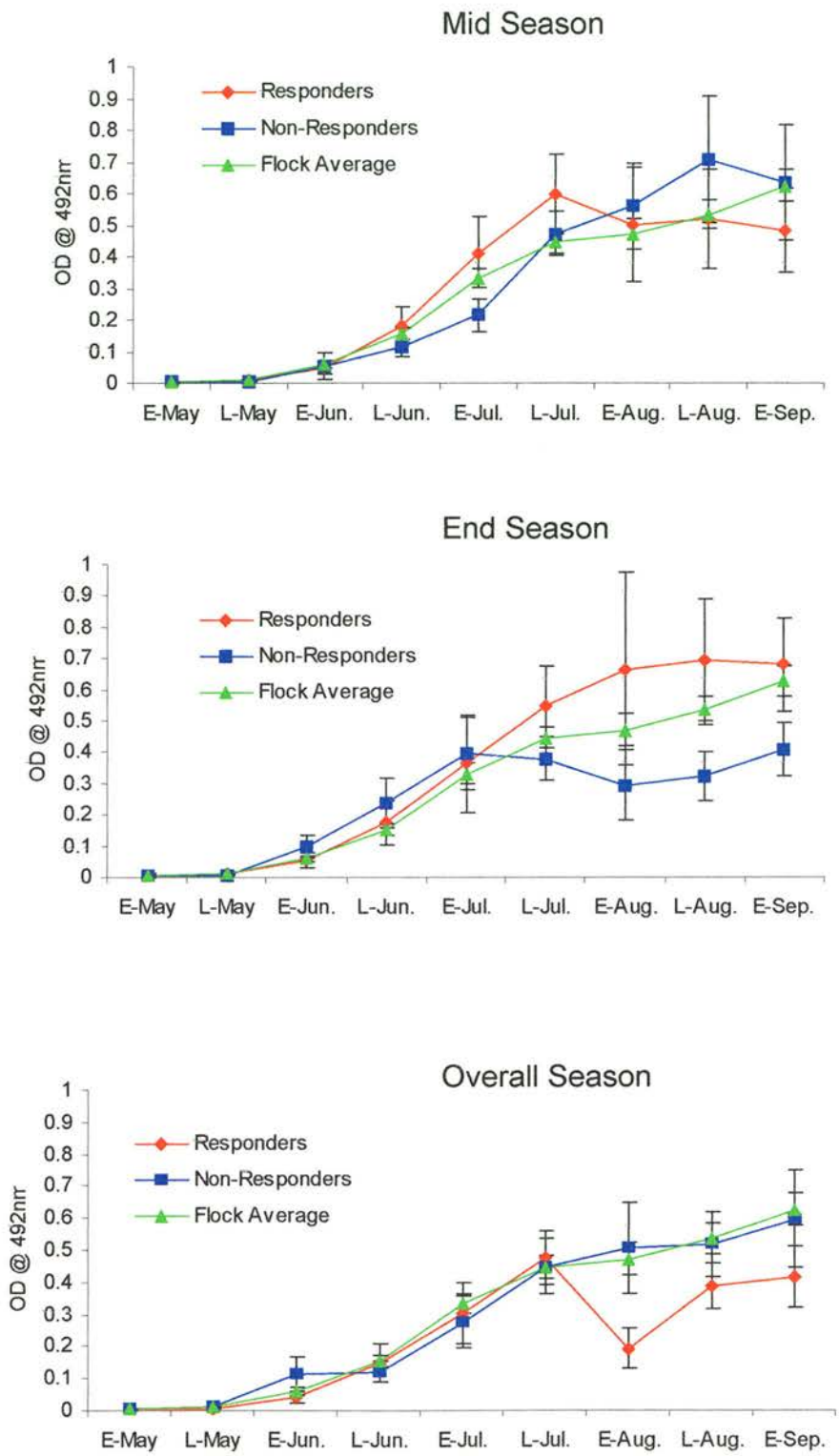


Figure 4.3 Average Parasite specific IgA Antibody levels (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

4.3.4 Correlations

4.3.4.1 FECs versus IgE antibody levels

The FEC and IgE antibody levels for the whole flock of lambs were ranked due to the presence of skewed data and then analysed to determine if any correlations existed between these two traits over the last three time points of the grazing season (8^{th} Aug.-6^{th} Sep.). The final part of the season was examined, as this was the time at which the lambs began to show a pronounced increase in antibody levels. The IgE antibody titres of the responders and non-responders selected using the lamb FEC rankings from the middle, end and overall part of the season were also analysed over the same time period to establish if the correlation with FECs was similar in these groups to the overall correlation. Table 4.3 displays the correlation results. Towards the end of the season, animals with higher FEC tended to have lower IgE levels in the whole flock ($\rho = -0.22$, $p=0.05$) and at this time period there was significant evidence of a strong linear relationship between the two variables. No strong associations were observed between FECs and IgE antibody concentration in any of the selected responder or non-responder lambs.

	Rho (ρ)	**P value**
Whole Flock	-0.22	0.05*
Responders		
Mid Season	-0.24	0.51
End Season	0.20	0.59
Overall Season	0.24	0.51
Non-Responders		
Mid Season	-0.21	0.56
End Season	0.18	0.63
Overall Season	0.55	0.10

Table 4.3 Correlations of the whole flock and selected responders and non-responders between FEC and IgE antibody titre over the last three time points

Plots were produced of the mean FEC against the mean immunological parameters measured of selected responders and non-responders over the last period of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean FEC and mean immunological factor of the whole flock of lambs, respectively. The lambs were located in different sections of the resultant quadrant. The raw data of each parameter was used to simply illustrate where animals might be found on the plot in relation to their FEC and immunological parameter. Figure 4.4 shows the four different sections and illustrates how the FEC and factor X correspond to each other, for example, the top left quadrant demonstrates a high FEC and low Factor X and the bottom left quadrant shows a low FEC and low Factor X etc.

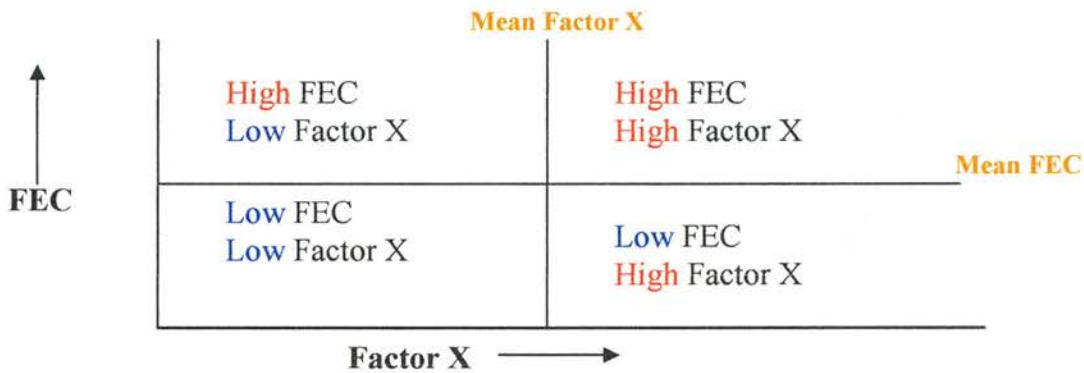


Figure 4.4 Schematic plot of FEC against Factor X

Figure 4.5 shows a plot of mean FEC versus mean IgE antibody level of the identified responders and non-responders over the last three time points of the grazing season. The whole flock mean of the FEC and IgE antibody was also illustrated on the graph. The mid season graph in Figure 4.5 showed that 60% of the non-responders were located in the low IgE and high FEC section and 50% of the responders were situated in the high IgE and low FEC section of the quadrant. The end season graph showed the same result with the non-responder lambs. However, only 40% of the responder lambs were situated in the high IgE and low FEC section of the quadrant. The overall season graph showed that all of the non-responder lambs were located in the low IgE and high FEC part of the quadrant and 50% of the responders were found in the high IgE and low FEC section.

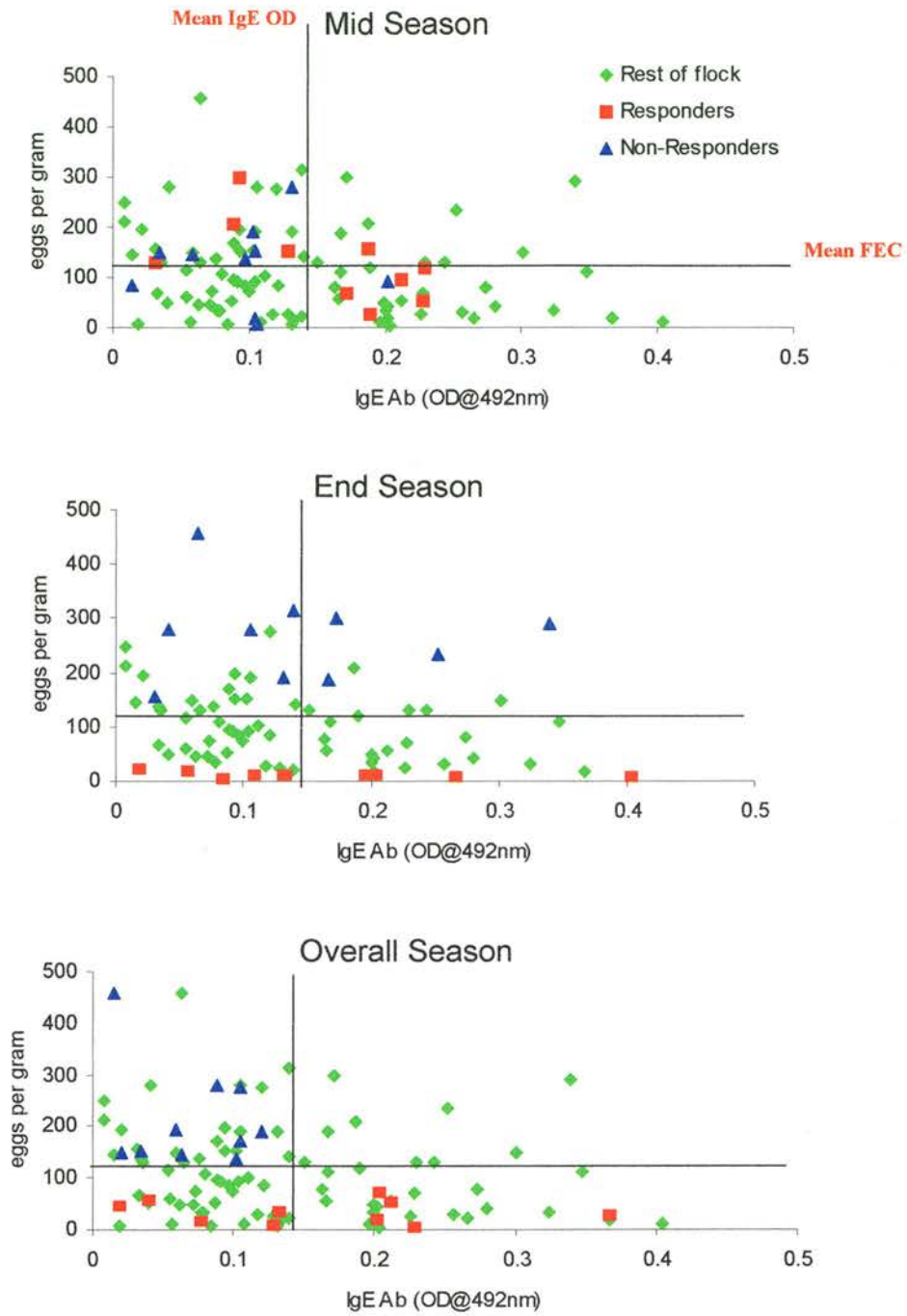


Figure 4.5 Plots showing mean FEC vs. mean IgE antibody titres of selected responders and non-responders using mid, end, and overall season FEC rankings for the last three time points of the grazing season

4.3.4.2 FECs versus IgA antibody levels

The FEC and IgA antibody levels were analysed in the same way as described in 4.3.4.1 to determine if any correlations existed between these two traits. Table 4.4 displays the correlation results. No correlations were determined within the whole flock or by any of the selected responder and non-responder animals.

	Rho (ρ)	P value
Whole Flock	-0.13	0.26
Responders		
Mid Season	-0.46	0.19
End Season	0.35	0.33
Overall Season	-0.08	0.83
Non-Responders		
Mid Season	-0.21	0.56
End Season	0.00	1.00
Overall Season	-0.22	0.53

Table 4.4 Correlations of the whole flock and selected responders and non-responders between FEC and IgA antibody titre over the last three time points

Figure 4.6 displays correlation plots showing the FEC levels and IgA antibody titre of the whole flock over the last three time points of the grazing season. As described in 4.3.4.1, the selected responders and non-responders using the mid, end and overall season FEC rankings were included on the three plots. Additionally, a vertical and horizontal line corresponding to the mean IgA OD and the mean FEC of the whole flock of lambs respectively was illustrated on the graphs. These two lines were used as described in 4.3.4.1 to determine which part of the quadrant the selected responders and non-responders were located in. In the mid season graph in Figure 4.6, only one responder was located in the bottom right quadrant corresponding to high IgA and low FEC and only 30% of the non-responders were found in the top left quadrant relating to low IgA and high FEC. The end season graph, however,

showed that 50% of the responders were present in the high IgA and low FEC section of the quadrant and 80% of the non-responders were located in the low IgA and high FEC section. In the overall season graph, 20% of the responders were found in the high IgA and low FEC part of the graph and 60% of the non-responders were situated in the low IgA and high FEC section.

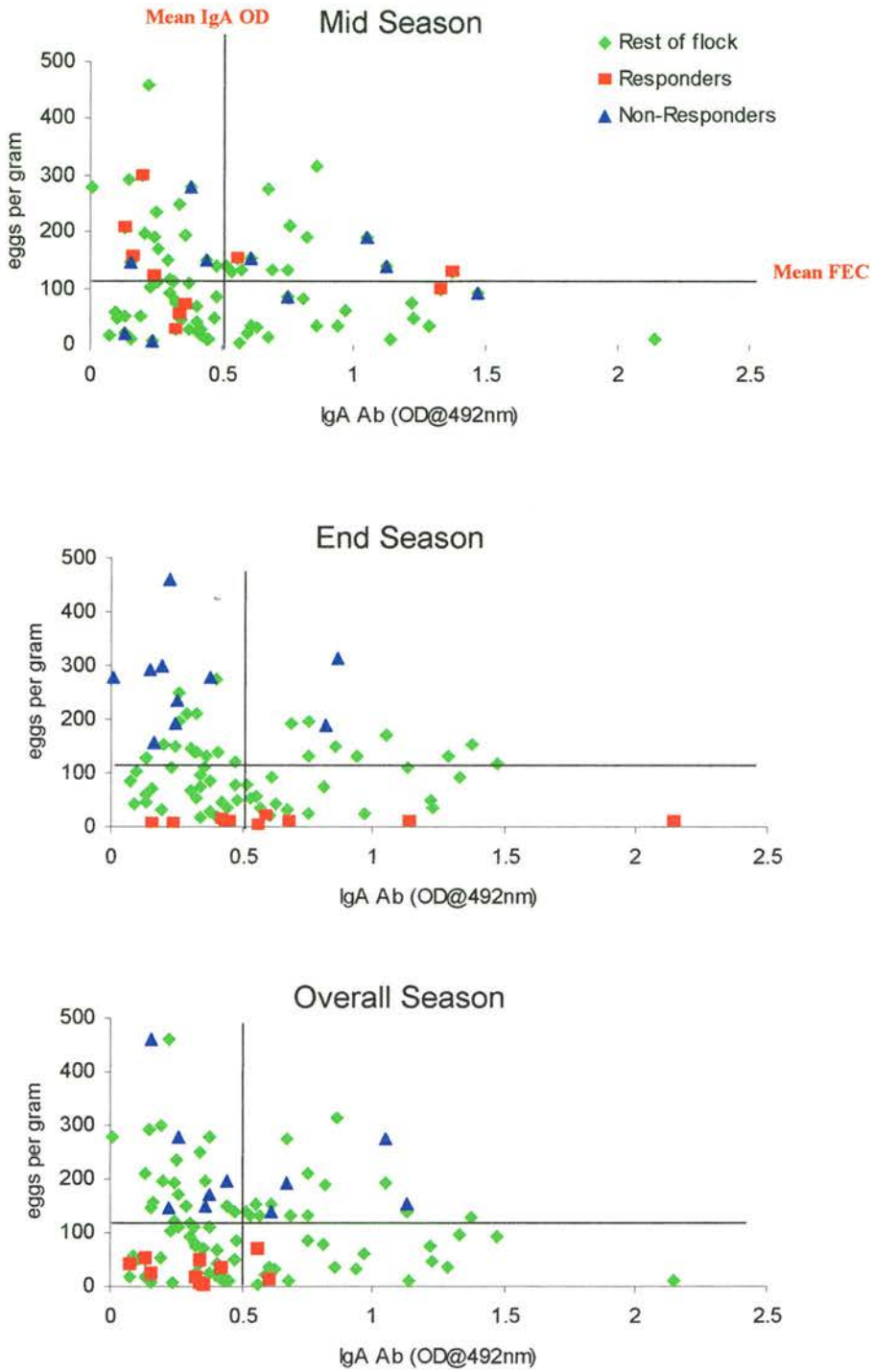


Figure 4.6 Plots showing mean FEC vs. mean IgA antibody titres of selected responders and non-responders using mid, end, and overall season FEC rankings for the last three time points of the grazing season

4.4 DISCUSSION

This study, provided evidence that the most effective time to select for responder and non-responder lambs using FECs was during the middle of the season at a time when the difference in egg count between the responder and non-responder lambs was significant. At this time, the non-responder lambs produced seven times more eggs than the responder lambs. Differences such as these have formed the basis for selection programmes throughout the world in sheep (Windon, 1996), goats (Patterson *et al.*, 1996) and cattle (Gasbarre *et al*, 2001). Differences in the pattern of egg count may be explained simply by differences in the rate of acquisition and expression of immunity. Responsiveness can be divided into three arbitrary, variable phases that are influenced not only by genotype but also by degree and extent of challenge and a variety of other factors such as nutrition, chemotherapy etc, the susceptible phase corresponding to a period when the animals have little or no immunity towards the gastrointestinal nematodes, the acquisition phase when animals begin to acquire an immune response against the parasite, and finally the expression phase when animals can maintain and express an effective immunity against the parasite. The schematic diagram in figure 4.7 illustrates how responsive sheep may acquire and begin to express their immunity to parasites at a faster rate than non-responsive sheep. However, although non-responsive sheep have significantly higher FECs during the acquisition phase, the majority of them may manage to eventually reach the same expression of immunity as the responsive sheep by the end of the season but at a slower rate.

Selecting responder and non-responder lambs during the expression phase which in this study occurred at the end of season, may lead to differences between the two groups of lambs being masked since during the expression phase many of the animals will have been able to acquire a similar level of immunity. For these reasons, it may be unwise to select responders and non-responders solely on the basis of FEC during the latter part of the season when there is evidence of the expression of acquired immunity.

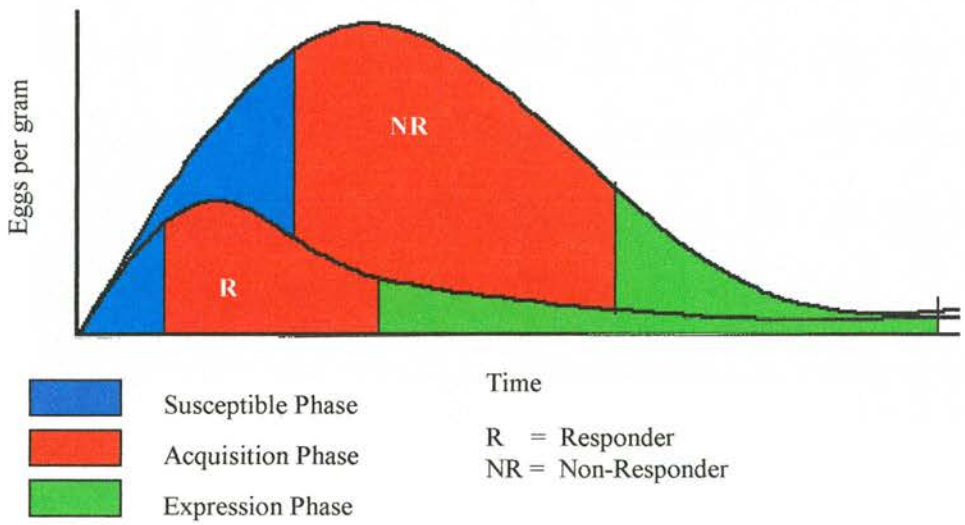


Figure 4.7 Schematic diagram to demonstrate three phases of responsiveness against nematodes in responsive and non-responsive animals

The parasite specific IgE and IgA antibody responses of both groups of lambs selected using the FEC rankings from all time points, only began to show a pronounced increase in antibody level from early July onwards and reached the highest concentrations during the end of the season when the FECs were generally low. This could be due to the antibody levels not being directly associated with FECs but being chronologically related with a lag phase appearing between egg output and antibody responses of lambs. Additionally, circulating antibody levels only measure the antibodies in the blood and may not provide a clear reflection of what is happening locally in the host. The local antibody response to nematode infection therefore may increase early on in the season when circulating antibody levels are negligible and consequently may be associated with FECs. Studies have reported that parasite specific IgE antibodies are generally higher in the lymph than in the serum (Huntley *et al.*, 1998b) indicating that the source of IgE is the local mucosa or draining nodes. Even though local immunity is likely to provide a clearer insight into the antibody responses that occur against nematode infection in sheep, it

would be impossible to employ local antibodies as markers of responsiveness to nematode infection simply because the collection of lymph involves cannulating the animals by surgical procedures. Douch *et al.*, 1996 has suggested that immunological parameters associated with nematode resistance that have the potential as phenotypic markers include circulating parasite-specific IgE and other studies have shown that circulating parasite specific IgA activity may help to identify lambs resistant to *T.circumcincta* (Strain *et al.*, 2002).

This study also demonstrated that responder lambs selected using the middle of the season FEC rankings had significantly elevated IgE levels compared to the non-responders over the last three time points of the grazing season. No significant differences in IgE titres were observed between the two groups of lambs selected using the FEC rankings from the end of the season. Additionally, the non-responder lambs selected using the mid season rankings showed that there was a tendency for these animals to have high FECs when the IgE levels were low towards the end of the season but these findings were not significant. Generally, there were no strong associations between FEC and IgE antibody responses of the identified responders and non-responders for each time point. The results suggest so far, however that it may be useful to select responders and non-responders based on late season IgE levels and earlier season FECs with the animals selected using the mid season rankings.

The parasite specific IgA levels displayed no significant differences between responders and non-responders selected using the FECs from any time point. Furthermore, no significant correlations were observed between IgA and egg output in both groups of lambs at any time point. Strain *et al.*, 2002 however, have demonstrated that Scottish blackface sheep, naturally infected with *T.circumincta* displayed an increase in IgA activity with a decrease in egg counts implying that peripheral IgA activitiy may help to identify lambs resistant to *T.circumcincta*. The IgA response however was measured against a somatic extract of 4th stage *T.circumcincta* larvae in Scottish Blackface sheep. These results therefore suggest that the specific parasitic stages of nematodes may have a profound effect on the type of antibodies being produced and on their relationships with egg output.

The ability to select lambs that respond to gastrointestinal nematode infection is very important, however, it may also be useful to select for non-responder animals. In this Greyface x Suffolk pilot study, the pasture was mainly contaminated with *T.circumcincta* and the results demonstrated that the flock of lambs produced on average 2143 million eggs across the whole of the grazing season. The responder lambs selected using the FECs from the middle of the season produced on average 173 million eggs accounting for only 8% of the total pasture contamination. The non- responder group of lambs, however produced 460 million eggs, which accounts for approximately 21%. A threefold difference in egg output was seen between these two groups of lambs selected from the middle of the season. The responder and non-responder lambs selected using the FECs from the end of the season produced on average 226 and 223 million eggs respectively, both groups of lambs accounting for approximately 10% of the pasture contamination. The selected non-responders from the overall season contributed to 23% of total pasture contamination, which was a six-fold higher egg output than the responder lambs that were only responsible for 4% of the pasture contamination. These results showed that the 10 identified non-responder animals were responsible for nearly one third of the pasture contamination, making these animals prime targets for anthelmintic treatment.

The results from this study were encouraging and suggested that circulating parasite specific IgE antibodies detected by ELISA may offer a rapid and sensitive means of measuring responsiveness against gastrointestinal nematodes in sheep based on responder and non-responders selected from the mid season FEC rankings. Additionally, selecting for non-responsive animals to nematode infection can be of value in reducing the amount of pasture contamination, and by treating only the selected non-responder lambs, anthelmintic usage can be minimised. Further studies will also attempt to measure the numbers of circulating IgE bearing cells in lambs naturally infected with *T.circumcincta* and evaluate their use as a marker of responsiveness. Circulating IgE bearing B lymphocytes, may migrate systemically before homing to parasite challenged mucosae in a manner similar to IgA bearing cells (Hall *et al.*, 1977). The detection and quantification of these may therefore provide a further assessment for the involvement of IgE antibody. Additional work will be carried out to determine if other immunological factors of responsiveness

such as eosinophils and IgE bearing cells could also be used as a marker of resistance against nematodes in sheep.

Chapter 5

Specific immunological parameters as markers of Responsiveness against *Teladorsagia* Infections in Scottish Blackface Lambs

2001 - 2002

5.1 INTRODUCTION

Studies undertaken in the previous chapter of this thesis and in other investigations have demonstrated the potential for identifying responder and non-responder animals based on their egg count (Patterson *et al.*, 1996; Baker *et al.*, 1990; Woolaston *et al.,* 1992; Windon, 1990).

The outcome of the pilot study using Greyface x Suffolk lambs has provided a foundation for the development of an immunologically based assay for selection of animals that are naturally resistant to nematode infection. The results, confirming previous observations that IgE is important in a host's resistance to parasite infection, need to be viewed in the context of previous literature, which documents the importance of IgA antibodies in resistance to *T.circumcincta*.

As this potential anomaly might be explained by differences in study design with respect to the breed of sheep studied (Greyface x Suffolk versus Scottish Blackface in Stear *et al.*, 1995a) and/or the particular *T.circumcincta* antigen used as the basis of the respective ELISAs (S300 purified L_3 versus L_4 and L_5) we sought to expand upon the studies undertaken in the previous chapters using a study design that would address these issues.

This study examined parasite specific IgE, IgA and IgG antibody titres, peripheral eosinophil levels, weight gains and circulating IgE bearing cells in Scottish Blackface lambs and importantly evaluated the relationship between responsiveness and performance.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Details of the Scottish Blackface lambs used in this study are described in 2.1.2. The lambs were put out on to pasture naturally infected predominantly with *T.circumcincta* at three weeks of age, from the end of May to late October. After 1 week (June 12th 2001) and 9 weeks (8th August 2001), all 98 sheep were treated with anthelmintic (Levacide 7.5%, Norbrook Animal Health, UK). Lambs were treated at the manufacturers recommended dose rate on the basis of liveweight. The sheep were monitored for 18 weeks initially and they were rehoused during the winter

months. During the second grazing season (March–October'02), animals were turned out on to the same pasture and were not treated with anthelmintic.

5.2.2 Parasitological Parameters

Rectal faecal samples of all animals were collected once fortnightly for the time they were on pasture. Faecal consistencies were monitored as described in 2.2.2. The number of eggs present in the faeces was determined using a flotation method as described in 2.2.3.

Lambs were ranked in order of their cumulative resistance to nematode infection as determined by their individual trichostrongylid faecal egg counts for the middle of the season (27th Jun-8th August'01) and also the end of the season (7th September-17th October'01). The ten lambs that had the lowest overall egg count were termed "responders" and the animals with the highest egg count were termed "non-responders". The average of the FEC rankings for each of these time periods was then calculated and an overall rank was given for each of the animals. The identified responders and non-responders selected from the first grazing season were followed for a second grazing season (2002).

5.2.3 Production Parameters

Lambs were weighed and bled once a month and serum was stored at –20°C for subsequent analysis (see 2.3).

5.2.4 Immunological Techniques

5.2.4.1 Parasite specific ELISAs using purified T.circumcincta L_3 antigen

Parasite specific IgE, IgA and IgG ELISAs were employed using the serum collected from the lambs over the first grazing season in 2001(see 2.4.2). ELISA plates were coated with S300 purified *T.circumcincta* L_3 antigen at 1µg/ml (see 2.4.2). The primary antibody (serum) was used in the IgE and IgA ELISA at a concentration of 1 in 10 and was used in the IgG ELISA at a concentration of 1 in 800. For the parasite specific IgE, IgA and IgG ELISAs the secondary antibodies

used were anti-IgE monoclonal, 2F1 at 1/1000 dilution, anti-IgA at 1/2000 dilution and anti-IgG at 1/2000 dilution, respectively.

In the second grazing season in 2002, the IgE ELISAs were carried out in the same way as described for the first grazing season. However, a new method was employed to improve the accuracy of the IgA and IgG assay.

Parasite specific IgA and IgG ELISAs in 2002 were carried out using the serum collected from the selected responder and non-responder lambs over the second grazing season (see 2.4.1). ELISA plates were coated as before with S300 purified *T.circumcincta* L_3 antigen at 1μg/ml (see 2.4.1). This new method, however, used eight dilutions of each serum sample (see table 5.1 for dilutions used for IgA and IgG ELISA). For the parasite specific IgA and IgG ELISAs the secondary antibodies used were anti-IgA and anti-IgG, respectively both at 1/2000 dilution. Once the ELISA was completed, the OD results for each dilution were plotted on a standard curve and the OD_{50} was evaluated to give a subsequent dilution titre.

Serum dilutions for IgA ELISA	Serum dilutions for IgG ELISA
1/2	1/10
1/5	1/50
1/10	1/100
1/50	1/500
1/100	1/1000
1/400	1/4000
1/1600	1/16000
1/12800	1/64000

Table 5.1 Serum dilutions for parasite specific IgA and IgG ELISAs

5.2.4.2 Eosinophil Counts

Once a month, the number of eosinophils present $X10^9$/litre in all of the selected lambs were determined as described in 2.4.3.

5.2.4.3 Determination of IgE bearing cells using Flow Cytometry

Fluorescence-activated cell sorter (FACS) analysis was used to determine the IgE bearing cells present within all the 98 Scottish Blackface lambs, and expressed as a % of the total immunoglobulin bearing cell population using the methods described in chapter 2.6.

5.2.5 Statistical Analysis

Statistical analysis was employed as described in 2.7. Differences between groups of lambs were compared using a two-tailed Student's t-test. Correlations between two variables were calculated using Spearman's rank correlation to see whether the two variables to be tested covary. $P<0.05$ was considered statistically significant in both statistical tests

5.3 RESULTS FOR FIRST GRAZING SEASON, 2001

5.3.1 Selected Responder and Non-Responder lambs using trichostrongylid egg counts

Table 5.2 shows the eartag numbers of the identified responders and non-responders using their trichostrongylid FEC rankings from the middle of the season, end of the season and the overall season.

Only two responder lambs (1012, 1099) and one non-responder lamb (978) were identified in both the mid season and end season rankings. Animal number 1104 was classed as a responder lamb during the middle of the season but was deemed a non-responder towards the end. The overall selected responder and non-responder animals consisted all together of five responder lambs and eight non-responder lambs from the animals selected using the mid and end season FECs.

Middle Season		End Season		Overall Season	
R	NR	R	NR	R	NR
653	746	740	631	653	631
781	834	744	779	679	719
826	908	850	783	699	898
945	909	852	861	744	908
960	910	857	877	755	909
967	916	1012	888	850	910
1012	941	1043	898	1012	916
1029	978	1082	924	1022	924
1099	1043	1099	978	1099	978
1108	1104	1104	1007	1115	1007

Table 5.2 Animals selected as responders and non-responders using their trichostrongylid FEC rankings from the mid, end and overall season

5.3.2 Faecal egg counts of selected responder and non-responder lambs using trichostrongylid FEC rankings

5.3.2.1 Trichostrongylid egg counts

The average trichostrongylid FECs of the selected responder and non-responder lambs from the three different time points over the grazing season are shown in Figure 5.1. The average FEC of the flock was included on each graph. The egg counts of the responder animals in the middle, end and overall periods followed a similar pattern across the grazing season. Their egg counts remained very low throughout, only peaking in late July to approximately 100 eggs per gram. The non-responders in the mid-season graph followed exactly the same pattern and only showed slight differences with the responder group of lambs from early July to early August. The non-responders in the end-season graph showed a higher egg count level compared to the responder lambs from early July to early August and this occurred again towards the end of the season. The non-responder animals reached egg count values of approximately 450 epg and 250 epg in the middle part of the season and towards the end, respectively. A difference between the egg counts of the two groups of lambs was also seen in the overall season graph with the non-

responders peaking in early August with a value of approximately 300 epg. In all three graphs in Figure 5.1, the faecal egg counts reached zero in both groups of lambs after anthelmintic treatment. The pasture that the Scottish Blackface lambs grazed on was mainly contaminated with *T.circumcincta*. The flock of lambs was estimated to have produced around 1719 million eggs across the grazing season. Table 5.3 shows the % of the total pasture contamination that the responders and non-responders selected from each time point were estimated to have produced during the grazing season.

	% Trichostrongylid Pasture contamination	
	Responders	**Non-Responders**
Mid Season	6	13
End Season	4	24
Overall Season	3	19

Table 5.3 Pasture contamination of responder and non-responder lambs over the grazing season

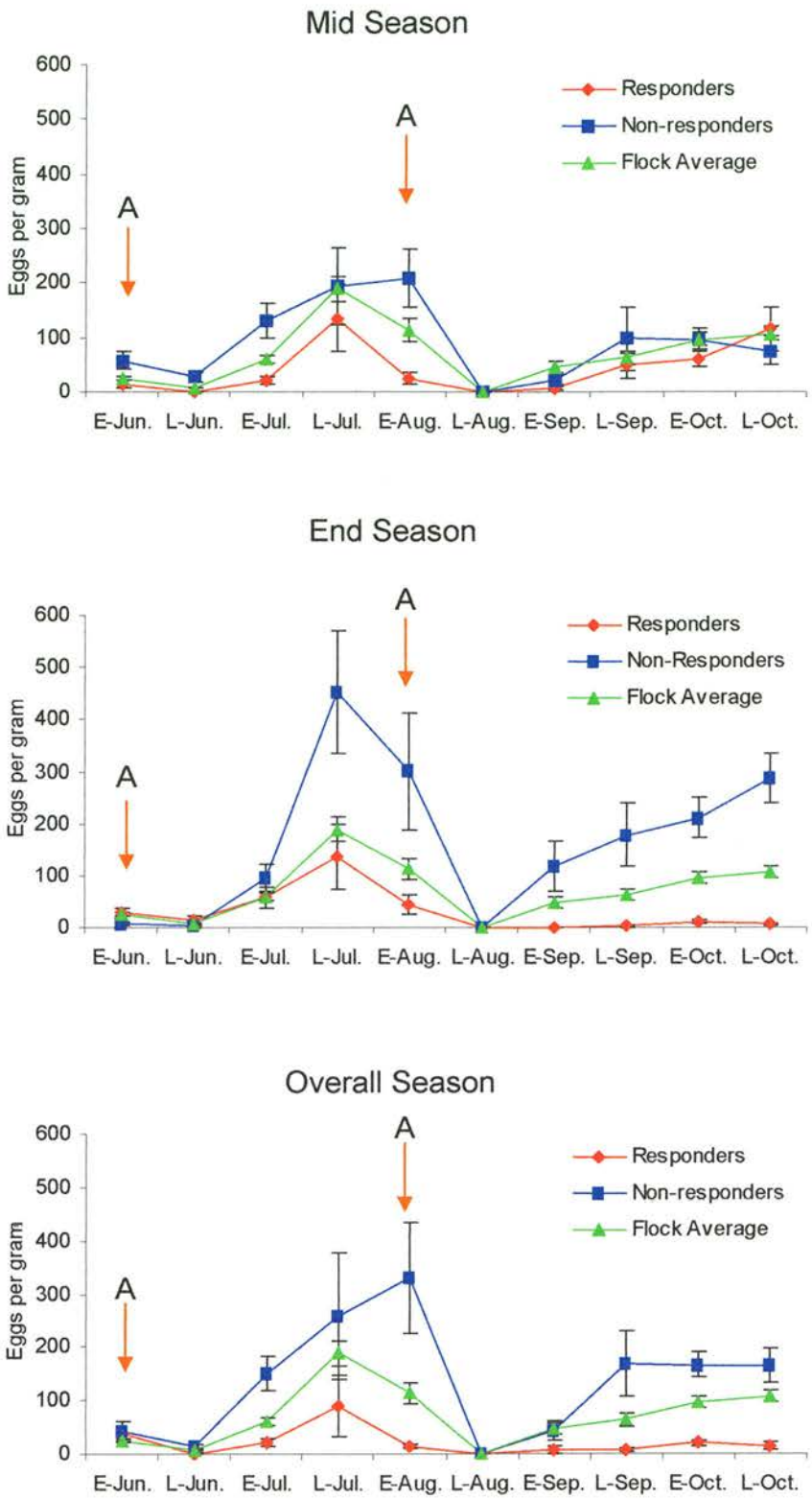


Figure 5.1 Average trichostrongylid faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using trichostrongylid FEC rankings

A = Anthelmintic treatment

5.3.2.2 Nematodirus *egg counts*

The average *Nematodirus* egg output for the selected responder and non-responder lambs using the trichostrongylid FEC rankings from different time points over the grazing season are demonstrated in Figure 5.2. The flock average egg count was included on each graph. All three graphs displayed a similar pattern in FECs for each group of lambs at each time point. The initial high *Nematodirus* egg output shown in all three graphs was controlled by the first anthelmintic treatment. From late June to late July there was a rise in egg count levels to approximately 400 epg in the non-responder lambs and approximately 150 epg in the responder lambs for each time point. Egg count levels then decreased to zero in late August and remained low for the rest of the season. Prior to the second anthelmintic treatment, *Nematodirus* egg counts were already beginning to decrease.

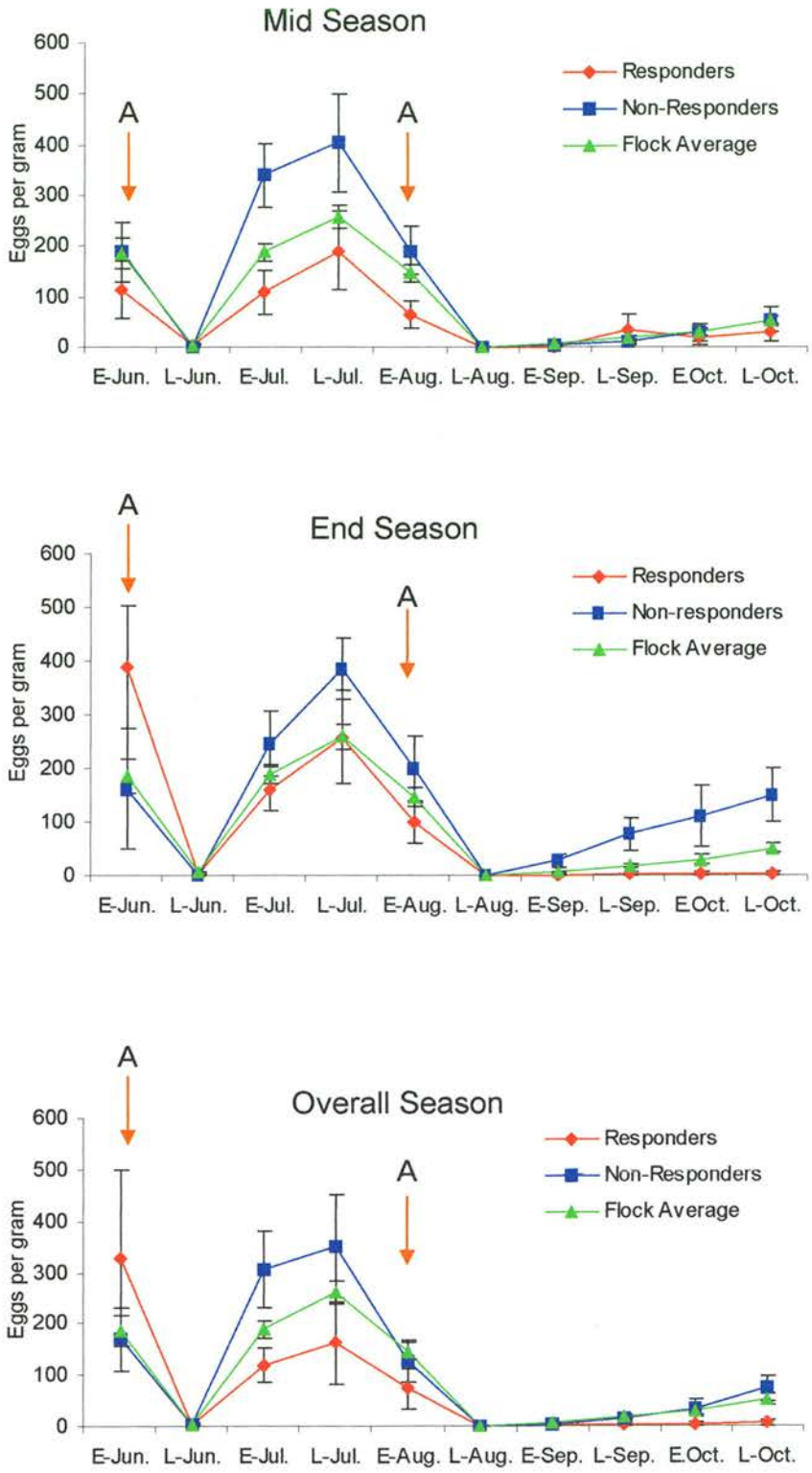


Figure 5.2 Average *Nematodirus* faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using trichostrongylid FEC rankings (A = Anthelmintic).

5.3.3 *Teladorsagia circumcincta* Specific ELISAs

5.3.3.1 IgE Antibody ELISAs

Figure 5.3 shows the average parasite (L_3) specific IgE antibody concentrations of the selected responder and non-responder lambs for the middle, end and overall grazing season using the trichostrongylid FEC rankings. In all of the graphs a general increase in IgE antibody response was observed across the season in both groups of lambs. No noticeable differences appeared between the responder and non-responder lambs selected from the end of the grazing season. A slight divergence in IgE levels between the responder and non-responder group of lambs from late August to late October in the mid season and overall season graphs was shown with the former group of lambs displaying a higher IgE antibody titre. These differences, however, were not statistically significant.

5.3.3.2 IgA Antibody ELISAs

The IgA antibody titres for the responder and non-responder groups of lambs selected using the trichostrongylid FEC rankings from the middle, end and overall grazing season are shown in Figure 5.4. The middle season graph showed a sudden increase in IgA antibodies from early July in both groups of lambs reaching an OD of approximately 1.0. No differences in IgA levels were observed between the responder and non-responder lambs across the grazing season and towards the end of the season the non-responders displayed higher IgA antibody concentrations than the responders. The IgA antibody concentrations in the end season and overall season graph followed a similar pattern to the mid season graph until early September, but towards the end of the season the IgA titres of the responder lambs were slightly higher than the IgA titres of the non-responder lambs. However, no significant differences in IgA levels were observed.

5.3.3.3 IgG Antibody ELISAs

Figure 5.5 displays the average parasite (L_3) specific IgG antibody levels for the selected responder and non-responder lambs using the trichostrongylid FEC rankings from different time points over the grazing season. The mid, end and overall season graphs all showed the same basic pattern in IgG levels over the course of the grazing

season. The IgG levels of both groups of lambs at all the time points did not begin to increase until late August. After this time point, the IgG titres increased quickly reaching OD levels of 1.0 by late October. The responder group of lambs displayed higher IgG levels than the non-responders from September onwards but the differences were not significant.

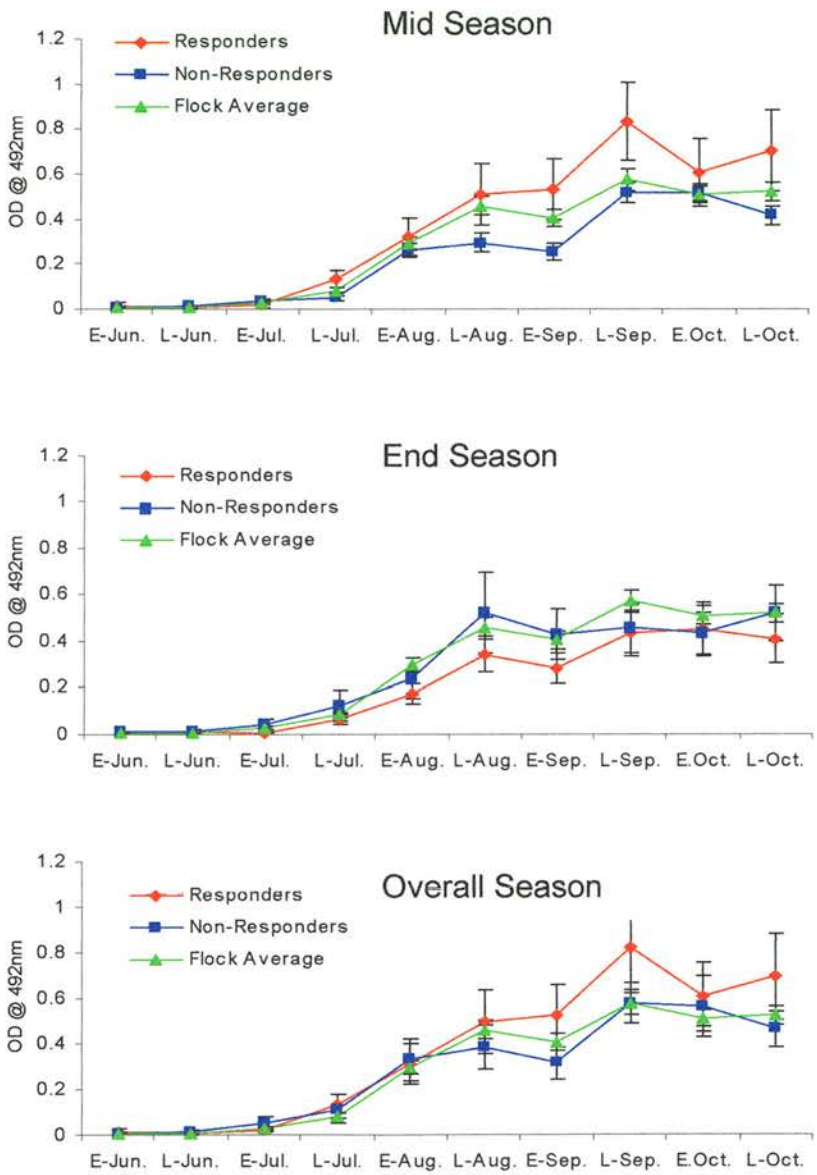


Figure 5.3 Average Parasite Specific IgE Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

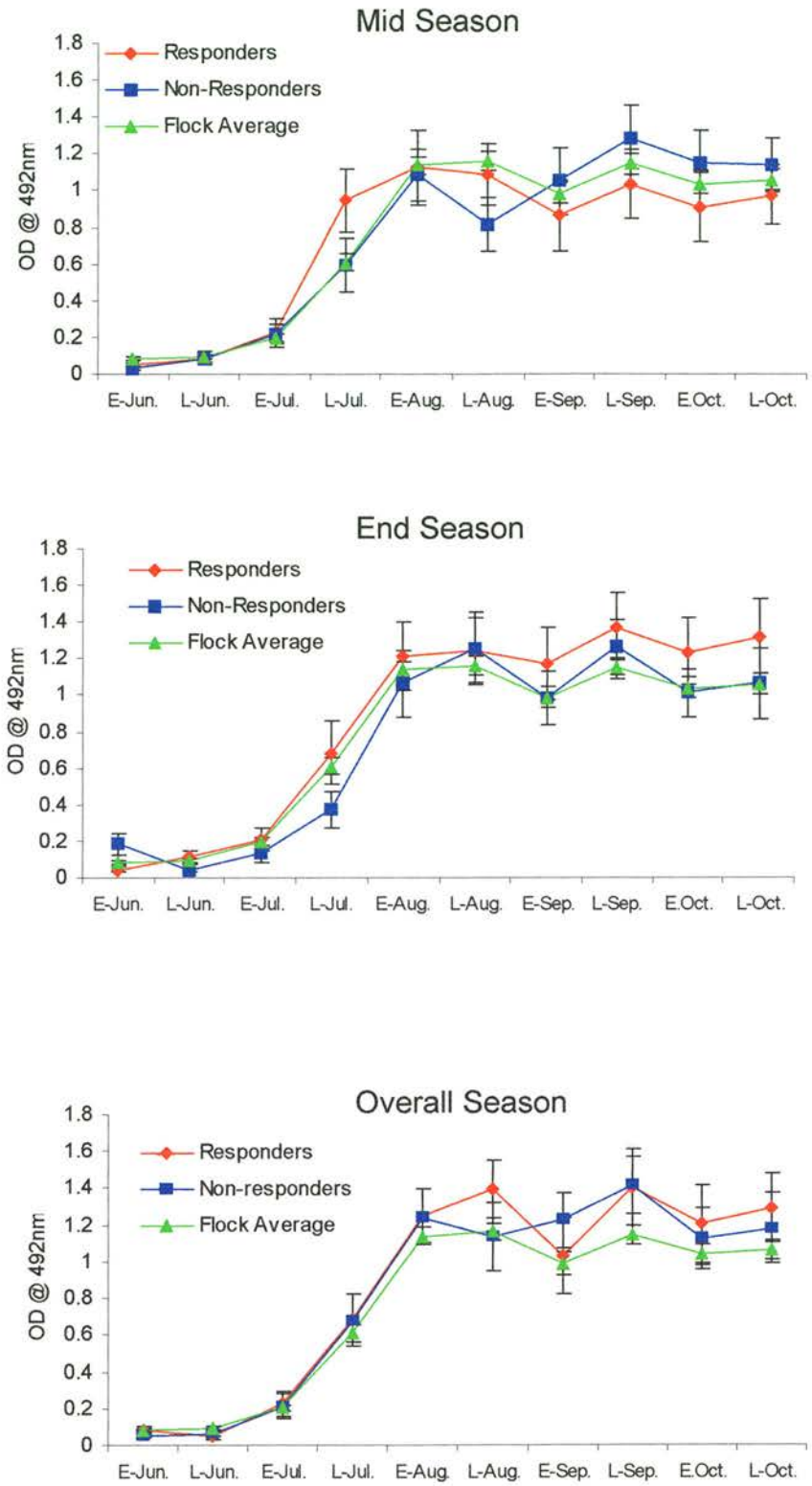


Figure 5.4 Average Parasite Specific IgA Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

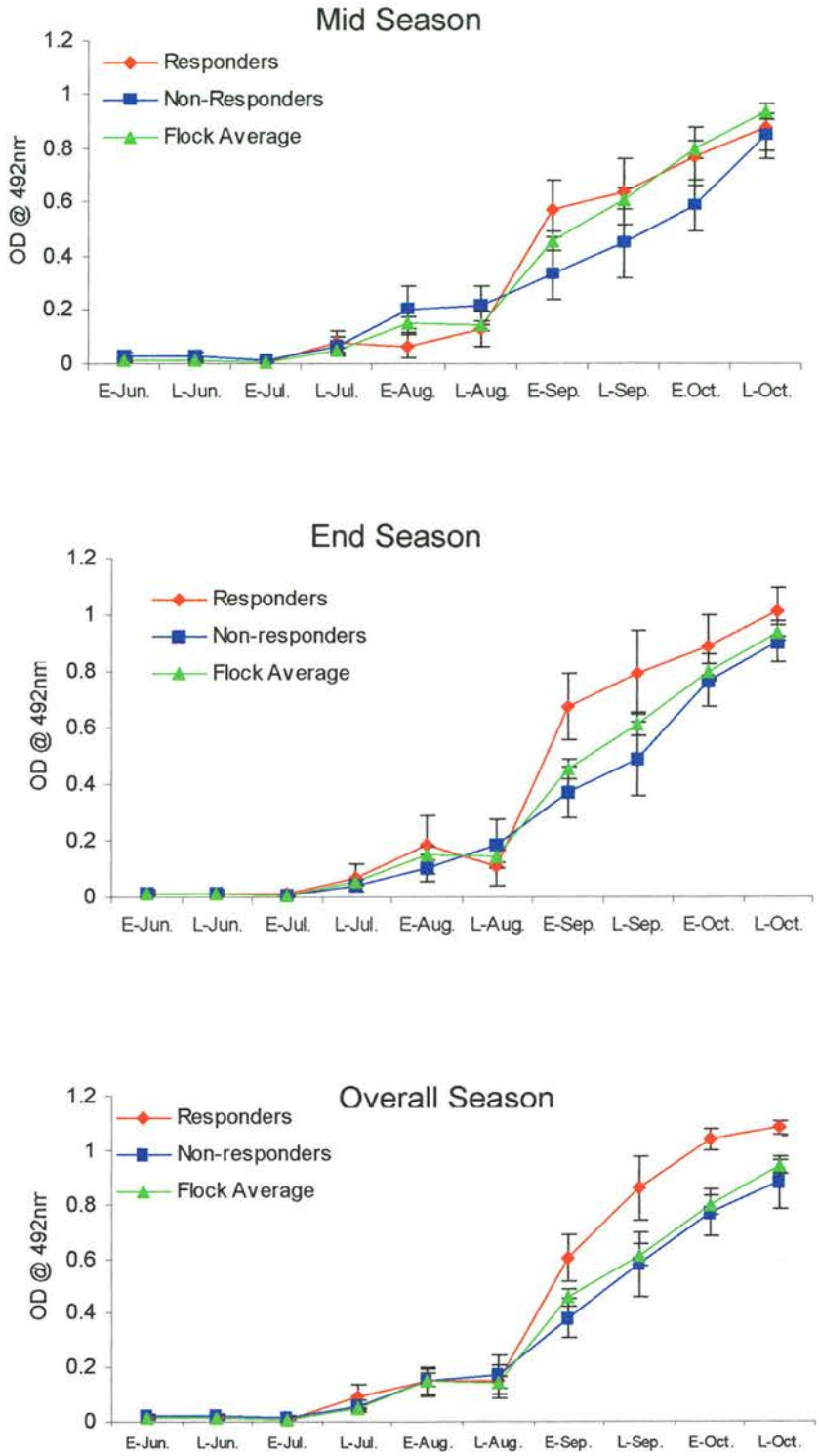


Figure 5.5 Average Parasite Specific IgG Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

5.3.4 Eosinophil counts of trichostrongylid selected responder and non-responder lambs

Figure 5.6 shows the average number of eosinophils x 10^9/litre for the selected responder and non-responder lambs using the trichostrongylid FEC rankings from the different time points over the grazing season. The mid and the end season graphs displayed similar numbers of eosinophils in both the responder and non-responder groups of lambs across the season. Over the middle part of the season (late June to early August) significant differences in eosinophil numbers in both the mid and end season graph were observed between the responders and non-responders ($p<0.05$ in both graphs) with the former group of animals demonstrating higher eosinophil numbers. No major differences between the two groups of lambs were observed in the overall season graph at any time point.

5.3.5 Weights of trichostrongylid selected responder and non-responder lambs

Figure 5.7 shows the average cumulative weight gain of the selected responder and non-responder animals using the trichostrongylid FEC rankings from different time points over the grazing season. All three graphs demonstrated a steady increase in weight from June to October. Responder lambs selected using the mid season and end season rankings gained significantly more weight than the non-responders during the last part of the grazing season ($p<0.01$, $p<0.05$, respectively). Additionally, the responders selected using the overall season rankings gained significantly more weight than the non-responders during the middle part and end of season ($p<0.05$, $p<0.01$, respectively).

5.3.6 IgE Bearing Cells of trichostrongylid selected responder and non-responder lambs

The three graphs shown in Figure 5.8 demonstrate the numbers of IgE bearing cells that were present in the selected responder and non-responder lambs using the trichostrongylid FEC rankings from the middle, end and overall grazing season. An increase in IgE bearing cell numbers of both groups of lambs was observed in all three graphs from August onwards. The numbers of IgE bearing cells of responder

lambs appeared to be higher than the cells of the non-responders, which was demonstrated clearly in the lambs selected from the middle of the season. A significant difference was seen at the last two time points (p=0.05).

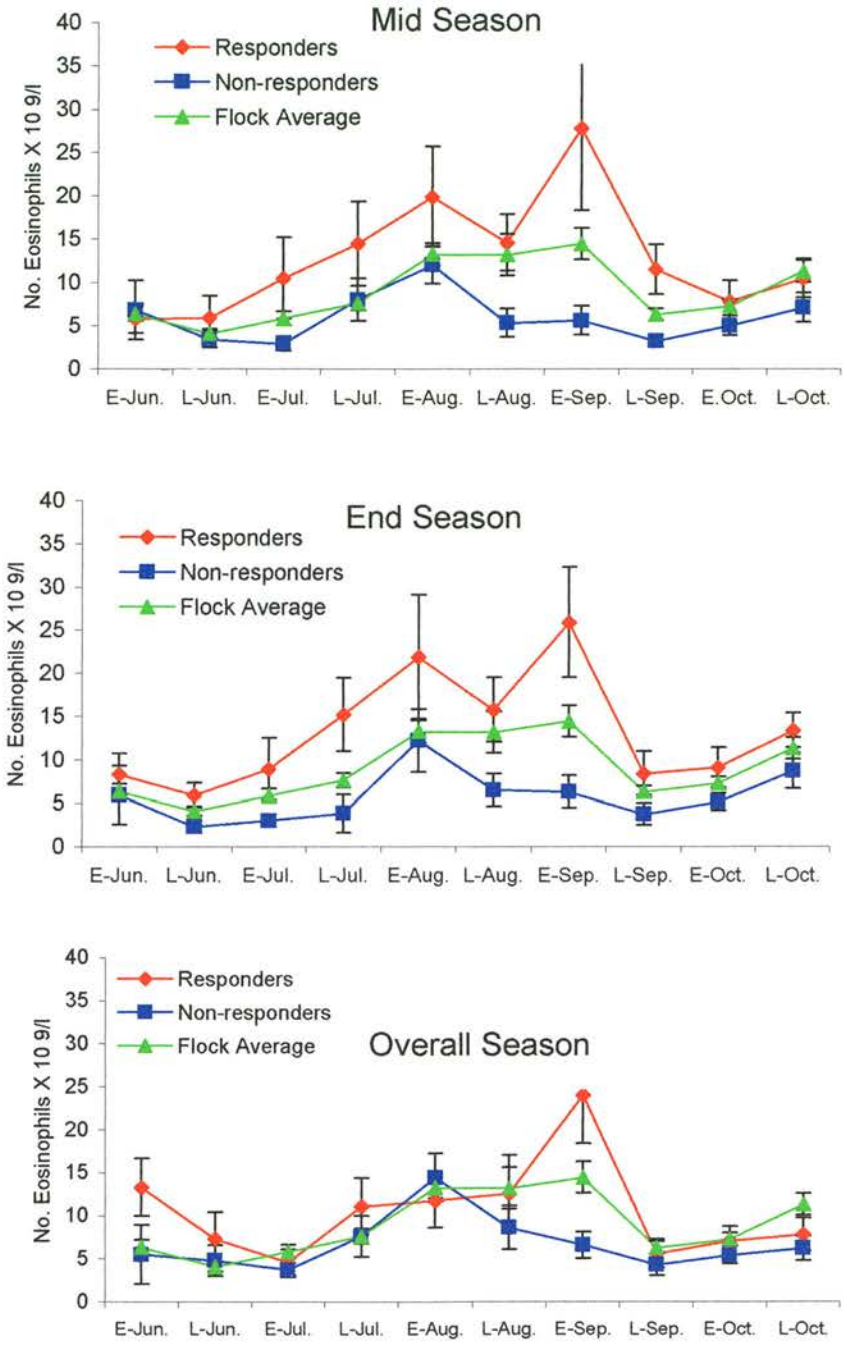
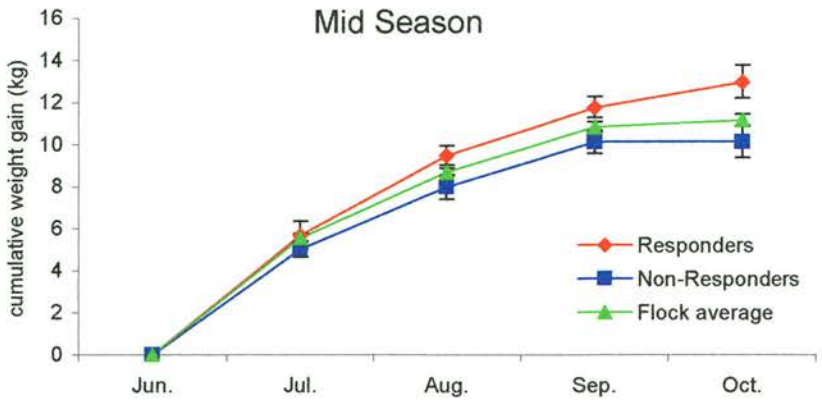
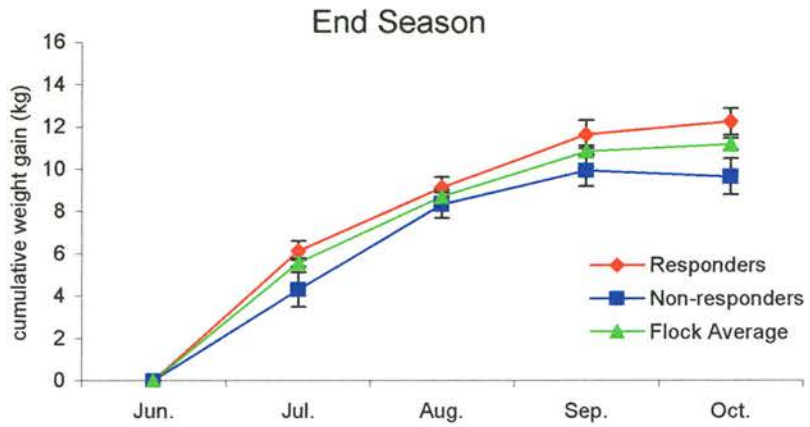


Figure 5.6 Average number of eosinophils x 10^9/l (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season





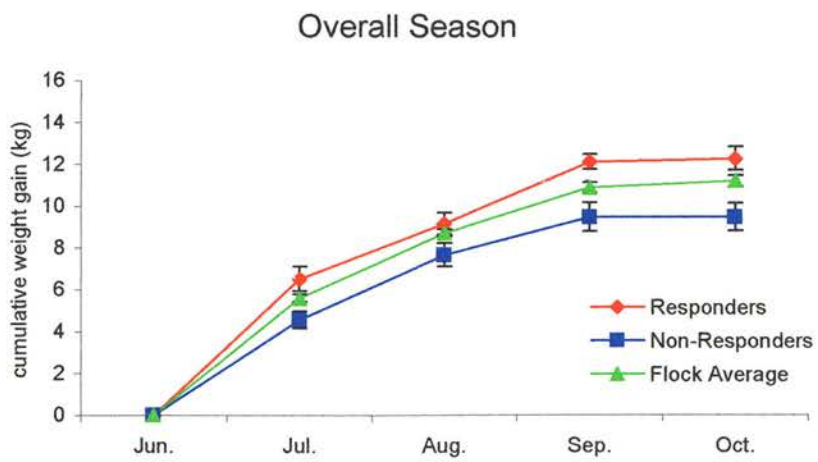
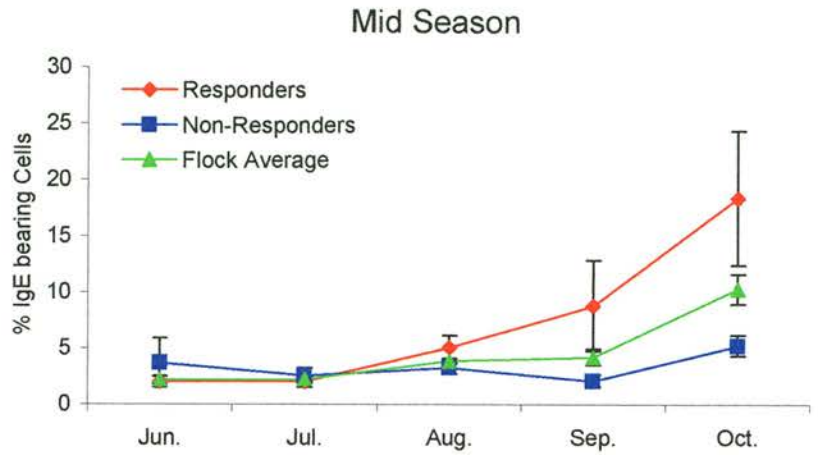
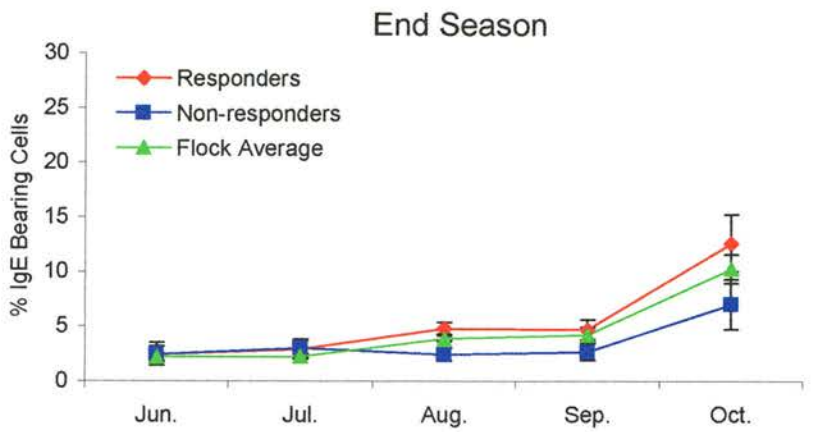


Figure 5.7 Average weight gain (kg) (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season





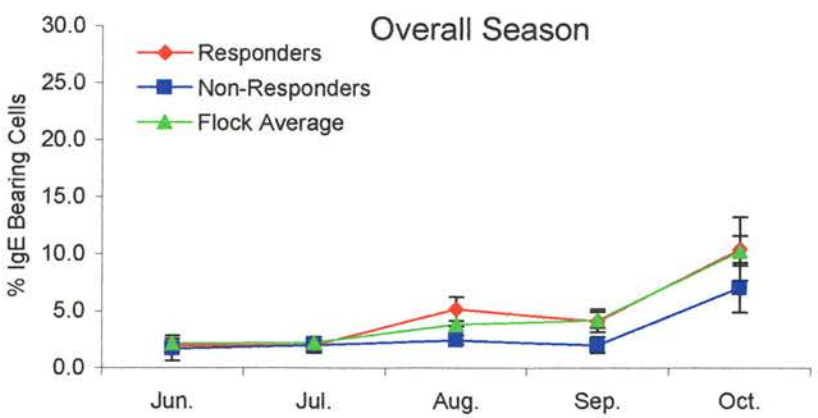


Figure 5.8 Average % IgE Bearing Cells (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season

5.3.7 The presence of IgE bearing cells from a typical trichostrongylid responder and non-responder lamb

IgE bearing cells identified from a typical trichostrongylid identified responder lamb towards the end of the season in the month of September by staining with anti-IgE-2F1 mAb are shown in Figure 5.9(a). A positive control and isotype control were included and the plots are illustrated in Figure 5.9 (b) and (c) respectively. A high % of IgE bearing cells were present in this responder lamb.

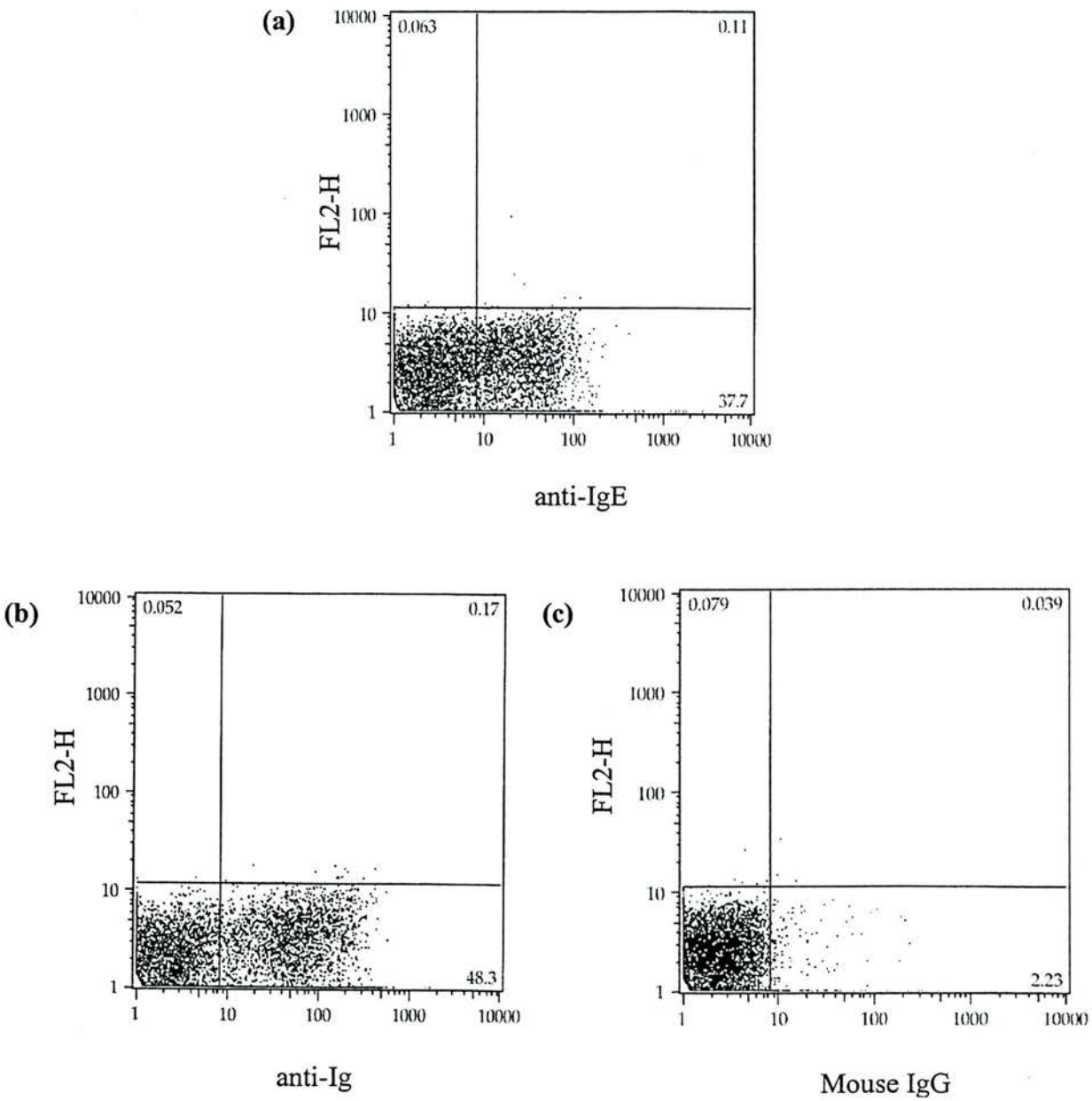


Figure 5.9 Number of IgE bearing cells present in a typical responder lamb

IgE bearing cells identified from a typical trichostrongylid identified non-responder lamb in the month of September are shown in Figure 5.10(a). A positive control and isotype control were included and the plots are illustrated in Figure 5.10 (b) and (c) respectively. A low % IgE bearing cells were found in this non-responder lamb.

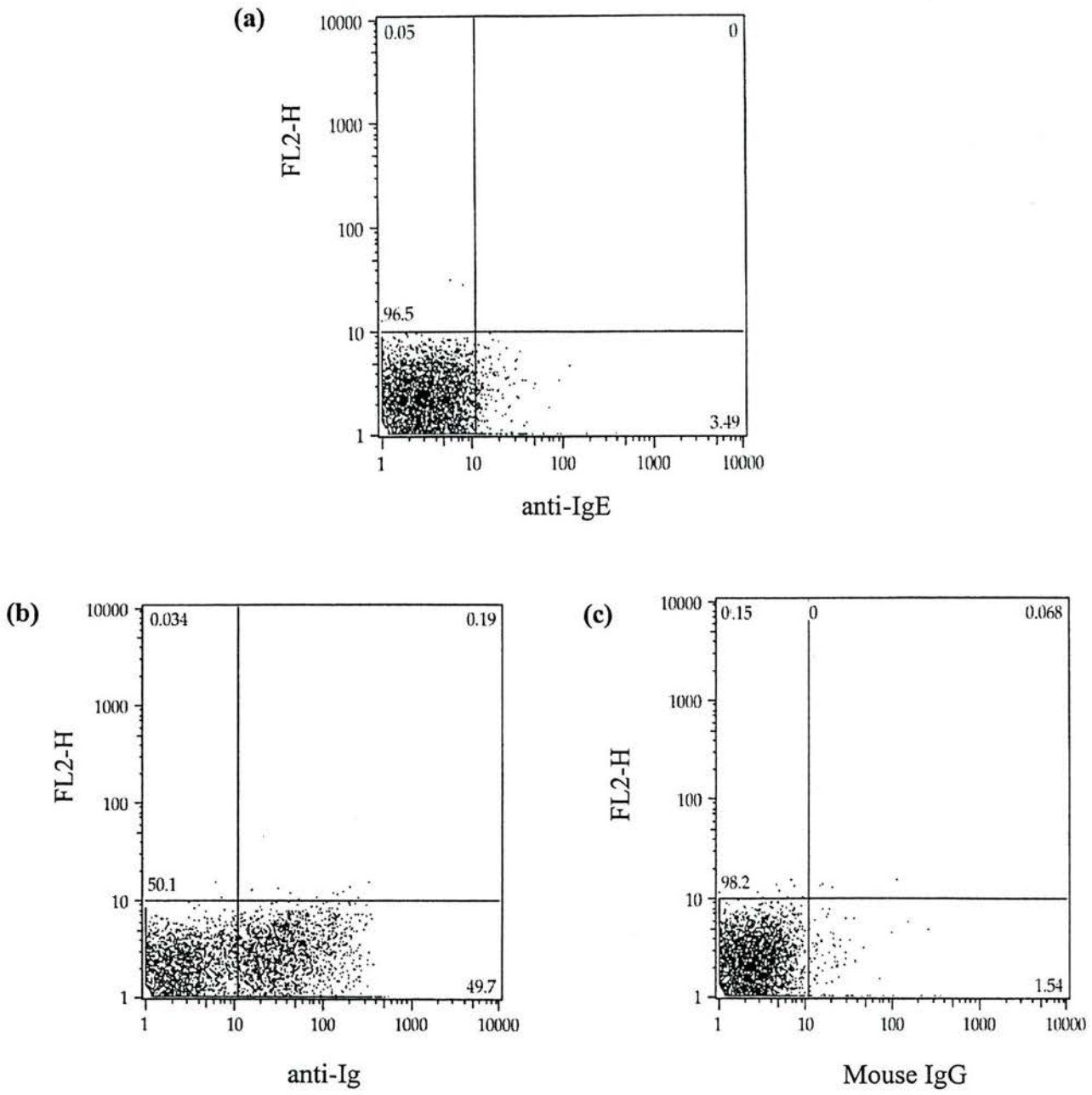


Figure 5.10 Number of IgE bearing cells present in a typical non-responder lamb

Figure 5.11 demonstrates that the majority of the IgE bearing cells found in the typical responder lamb were located among the lymphocytes and granulocytes. However, the proportion of IgE bearing cells was much larger in the lymphocyte population in all samples analysed.

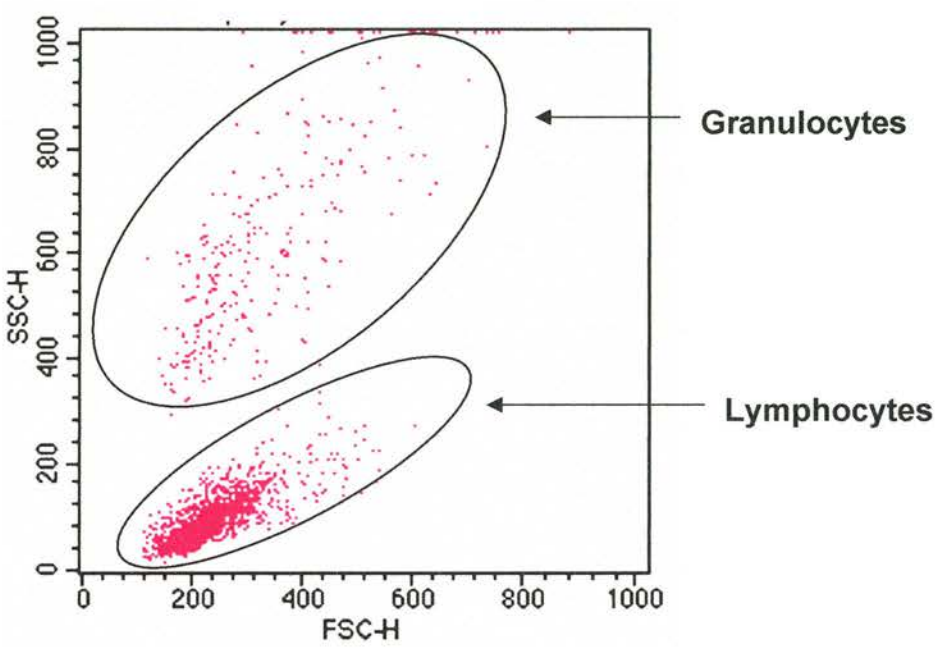


Figure 5.11 Location of IgE Bearing Cells

5.3.8 Trichostrongylid Correlations

5.3.8.1 Trichostrongylid *FECs versus IgE, IgA and IgG antibody levels*

The trichostrongylid FEC and antibody levels for the whole flock of lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season (7th Sep.-17th Oct'01). The final period of the season was examined, as this was the time at which the lambs began to show a pronounced increase in antibody levels. The antibody titres of the responders and non-responders selected using the lamb FEC rankings from the middle, end and overall part of the season were also analysed over the same time period to establish if

they also shared any association with their FECs. Tables 5.4-5.6 display the correlation results between FEC and IgE, IgA and IgG antibody levels, respectively.

No correlations were observed between FECs and IgE antibody concentration in the whole flock or in any of the selected responder or non-responder lambs during the end of the season.

Within the whole flock over the last four time points, there was significant evidence that lambs with higher FECs tended to have lower IgA levels (ρ=-0.23, $p<0.05$). Additionally, the responder lambs selected using the mid season FEC rankings demonstrated low egg output and high IgA antibody titres (ρ=-0.61, $p=0.06$). No significant correlations were observed by any of the other selected responder and non-responder animals during the end of the grazing season.

Table 5.6 displays the correlation results for the FECs and IgG antibody levels for the end of the grazing season in the whole flock and identified responders and non-responders. No correlations were observed between these two traits in any of the lambs during the end of the season.

	Rho (ρ)	P value
Whole Flock	0.06	0.54
Responders		
Mid Season	0.30	0.41
End Season	0.26	0.47
Overall Season	0.33	0.35
Non-Responders		
Mid Season	0.12	0.75
End Season	-0.10	0.79
Overall Season	-0.07	0.85

Table 5.4 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and IgE antibody titre over the last four time points

	Rho (ρ)	P value
Whole Flock	-0.23	0.03*
Responders		
Mid Season	-0.61	0.06
End Season	-0.35	0.32
Overall Season	-0.04	0.91
Non-Responders		
Mid Season	0.30	0.39
End Season	0.11	0.76
Overall Season	-0.10	0.79

Table 5.5 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and IgA antibody titre over the last four time points

	Rho (ρ)	P value
Whole Flock	-0.11	0.29
Responders		
Mid Season	-0.51	0.13
End Season	-0.32	0.37
Overall Season	-0.52	0.13
Non-Responders		
Mid Season	0.21	0.56
End Season	0.29	0.41
Overall Season	-0.06	0.87

Table 5.6 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and IgG antibody titre over the last four time points

5.3.8.2 Trichostrongylid *FECS versus eosinophils*

The trichostrongylid FEC and eosinophil numbers for the whole flock of lambs were analysed to determine if any correlations existed between these traits over the

last four time points of the grazing season. The eosinophil numbers of the responders and non-responders selected using the trichostrongylid FEC rankings from the middle, end and overall part of the season were also analysed over the same time period to establish if they also shared any association with their FECs. Table 5.7 displays the correlation results between FEC and the numbers of eosinophils x 10^9/litre. No correlations were observed between FECs and eosinophil numbers in the whole flock. Identified mid season responder animals with lower FEC tended to have higher eosinophil numbers (ρ=-0.5, p=0.14) but during this time period there was no evidence of a strong relationship between the two variables. The end season responder lambs showed this same pattern of lower FECs and higher eosinophil numbers, however, these animals did show a significant relationship between FEC and eosinophil numbers (ρ=-0.64, p<0.05). The overall season responder lambs showed no correlation between these two traits. The non-responder lambs selected using the mid season rankings showed a significant association between these two parameters with low eosinophil levels and an increased egg output (ρ= -0.68, p<0.05). The non-responders selected using the end and overall season rankings showed no significant correlations between FEC and eosinophil numbers.

	Rho (ρ)	**P value**
Whole Flock	-0.12	0.25
Responders		
Mid Season	-0.50	0.14
End Season	-0.64	0.04*
Overall Season	-0.30	0.40
Non-Responders		
Mid Season	-0.68	0.03*
End Season	-0.16	0.66
Overall Season	-0.07	0.84

Table 5.7 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and eosinophil numbers over the last four time points

Using the same procedure as in the previous study (Chapter 4), plots were produced of the mean FEC against the mean immunological or production parameters measured in selected responders and non-responders over the last period of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean FEC and mean immunological or production factor of the whole flock of lambs, respectively. The lambs were located in different sections of the resultant quadrant. The raw data of each parameter was used to simply illustrate where animals might be located on the plot in relation to their FEC and immunological or production trait.

Figure 5.12 shows a plot of mean FEC versus mean eosinophil number of the identified responders and non-responders over the last four time points of the grazing season. The whole flock mean of the FEC and eosinophil counts was illustrated on the graph. The mid season graph in Figure 5.12 showed that 20% of the responders were located in the low FEC and high eosinophil count section of the graph and 50% of the non-responders were found in the high FEC and low eosinophil count section. The end season graph showed that 80% of the responders were found in the top left quadrant relating to low FECs and high eosinophil counts, however, the non-responders were mainly found in the bottom left quadrant corresponding to a low FEC and low eosinophil count. The overall season graph demonstrated that 30% of the responders were located in the low egg count and high eosinophil number section and the non-responders were present in the low FEC and low eosinophil number section.

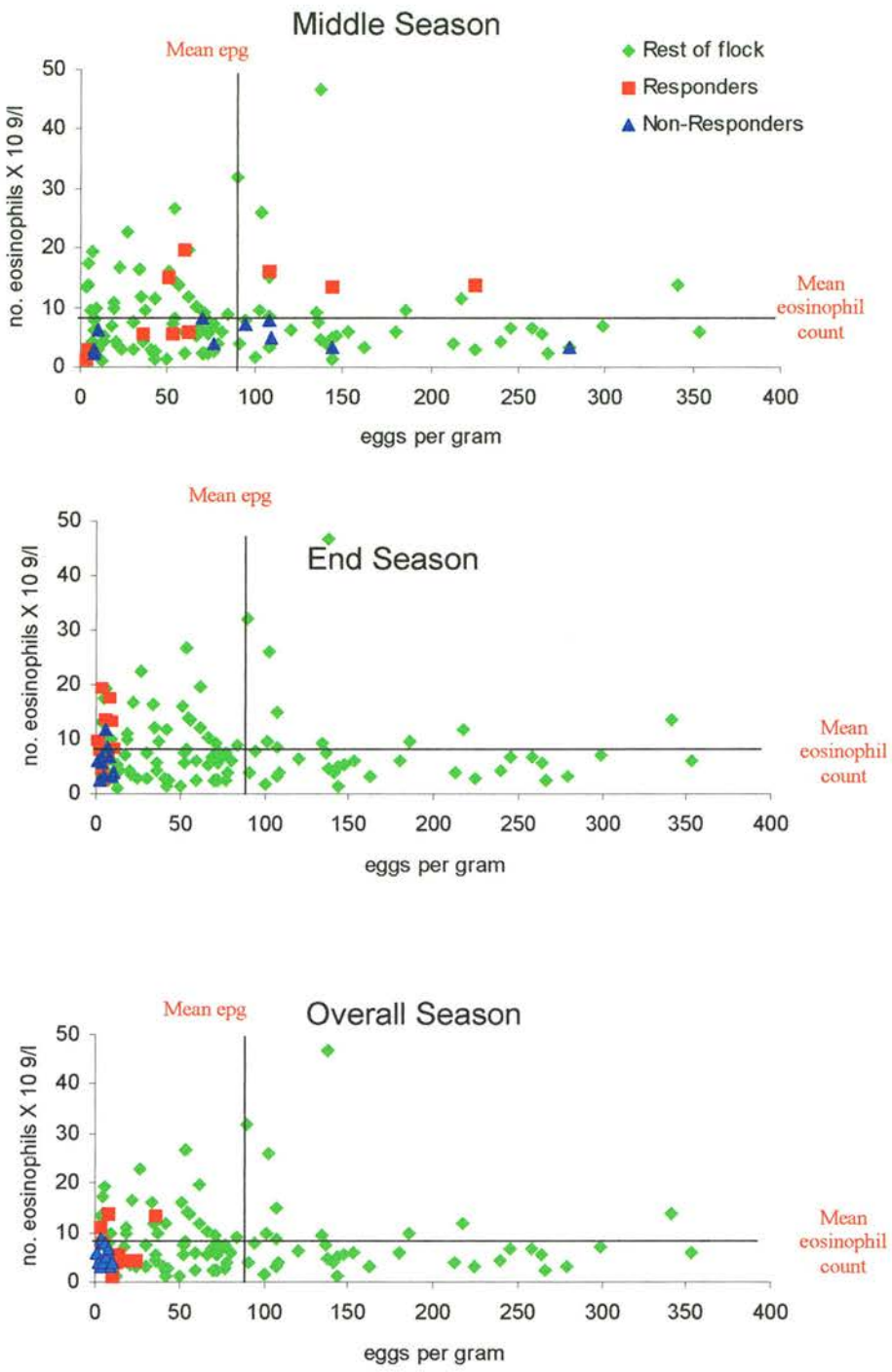


Figure 5.12 Plots showing mean trichostrongylid FEC vs. mean eosinophil number of selected responders and non-responders for the last four time points of the grazing season

5.3.8.3 Trichostrongylid *FECS versus cumulative weight gain*

The trichostrongylid FEC and cumulative weight gain for the whole flock of lambs and for the identified responder and non-responder lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season. Table 5.8 displays these correlation results. Within the whole flock during the end of the season, animals that gained more weight tended to have lower FEC levels and there was significant evidence of a relationship between these two variables (ρ=-0.34, p=0.001). No correlations were observed between FECs and cumulative weight gain in any of the identified responder and non-responder animals.

	Rho (ρ)	P value
Whole Flock	-0.34	0.001*
Responders		
Mid Season	0.17	0.64
End Season	-0.32	0.36
Overall Season	-0.03	0.93
Non-Responders		
Mid Season	-0.23	0.52
End Season	0.02	0.95
Overall Season	-0.05	0.89

Table 5.8 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and cumulative weight gain over the last four time points

Figure 5.13 demonstrates the graph of mean FECs against mean cumulative weight gain of identified responder and non-responder lambs towards the end of the grazing season. The vertical line and horizontal lines on the graph represents the mean FEC and mean cumulative weight gain of the whole flock, respectively. The mid season graph in Figure 5.13 showed that 60% of the responders were distributed in the low FEC and high weight gain section and only 30% of the non-responders were situated in the high FEC and

low weight gain section. In the end season and overall season graphs, 80% of the responders were present in the low FEC and high weight gain section of the graph and 80% of the non-responders were present in the high FEC and low weight gain section.

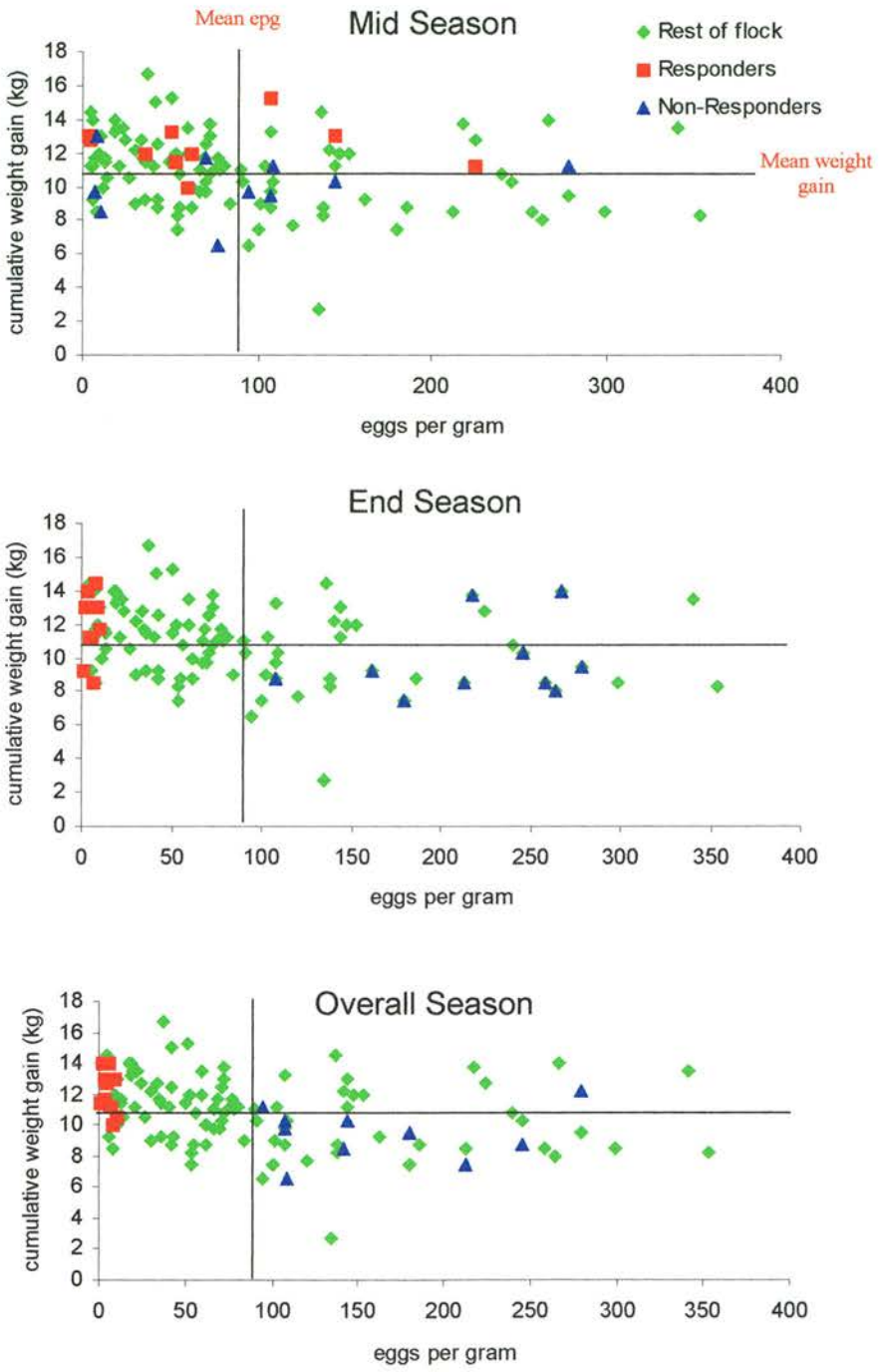


Figure 5.13 Plots showing mean trichostrongylid FEC vs. mean cumulative weight gain of selected responders and non-responders for the last four time points of the grazing season

5.3.8.4 Trichostrongylid *FECS versus IgE bearing cells*

The trichostrongylid FEC and numbers of IgE bearing cells for the whole flock of lambs and for the identified responder and non-responder lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season. Table 5.9 displays these correlation results. Within the whole flock during the end of the season, animals with high FEC levels tended to have lower IgE bearing cells and there was significant evidence of a relationship between these two variables (ρ= -0.20, $p<0.05$). The identified mid season non-responders demonstrated that FEC levels tended to be high when IgE bearing cell numbers were low yet there was no significant evidence of a relationship between these two variables (ρ= -0.49, p=0.15). Additionally, the identified overall season responder lambs tended to have low FECs and high IgE bearing cell numbers but these results were not significant (ρ= -0.56, p=0.09). No correlations between these two traits were demonstrated in any of the other lambs.

	Rho (ρ)	P value
Whole Flock	-0.20	0.05
Responders		
Mid Season	-0.20	0.58
End Season	-0.41	0.24
Overall Season	-0.56	0.09
Non-Responders		
Mid Season	-0.49	0.15
End Season	-0.20	0.58
Overall Season	-0.01	0.99

Table 5.9 Correlations of the whole flock and selected responders and non-responders between trichostrongylid FEC and IgE bearing cells over the last four time points

Figure 5.14 demonstrates the plot of mean FECs against mean IgE bearing cells of identified responder and non-responder lambs towards the end of the grazing season. The vertical line and horizontal lines on the graph represents the mean FEC and mean IgE bearing cells of the whole flock, respectively. In all three graphs (Mid, End and Overall) in Figure 5.14, 40%, 70% and 40% of the responders were distributed in the low FEC and high IgE bearing cell part of the graph respectively and 50%, 60% and 80% of the non-responders were situated in the high FEC and low IgE bearing cell section, respectively.

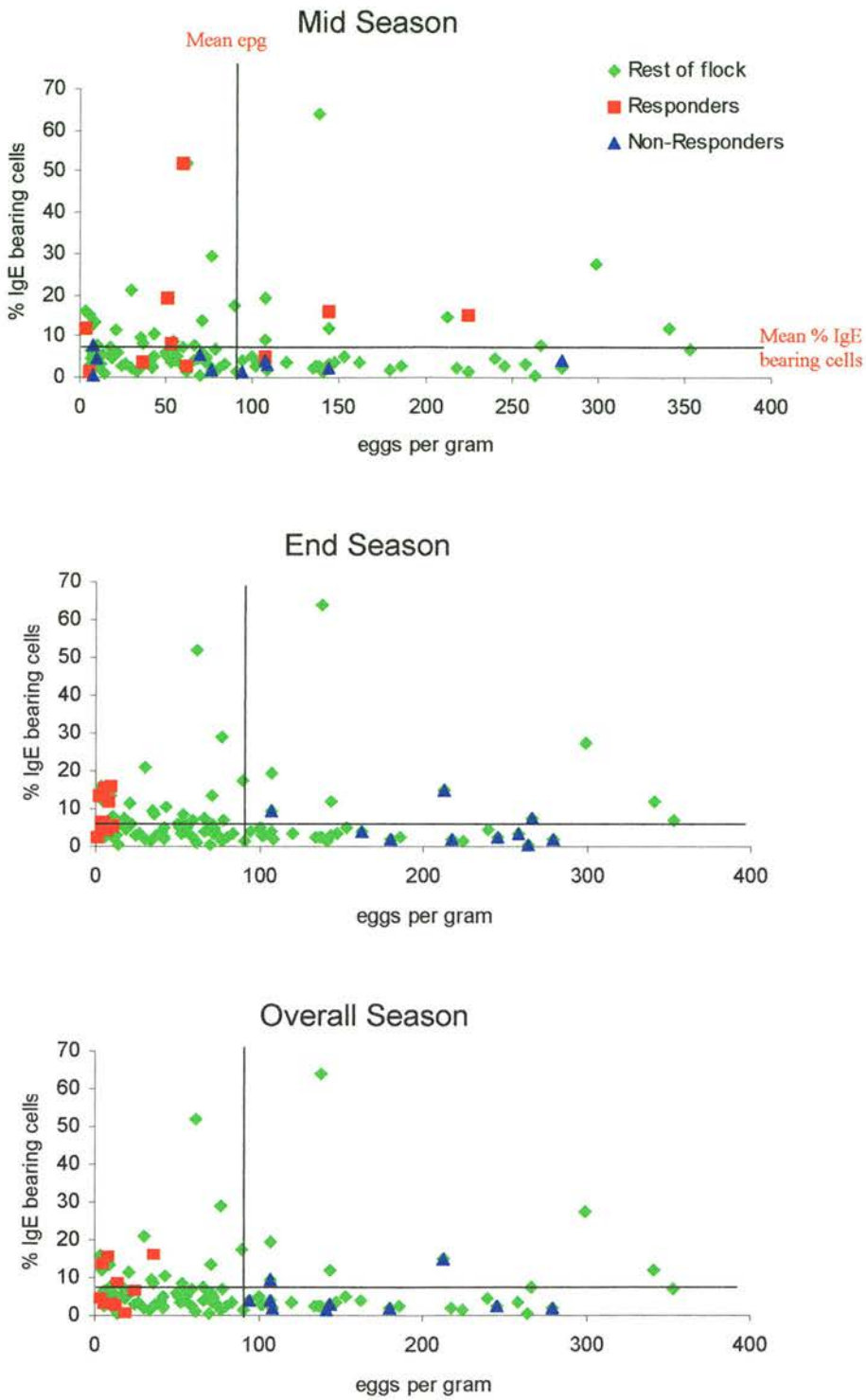


Figure 5.14 Plots showing mean trichostrongylid FEC vs. mean IgE bearing cells of selected responders and non-responders for the last four time points of the grazing season

5.3.9 Cumulative weight gain versus immunological parameters for responders and non-responders selected using trichostrongylid FEC rankings

It was additionally important to establish if any of these immunological parameters had a strong association with the cumulative weight gain of the identified responder and non-responder lambs as selecting for a high growth in lambs is just as important as selecting for lambs with low FEC. Correlations were therefore evaluated for cumulative weight gain against eosinophil numbers, and IgE bearing cells in the trichostrongylid selected responders and non-responders.

5.3.9.1 Cumulative weight gain versus eosinophils for lambs selected using trichostrongylid *FECs*

Table 5.10 demonstrates the correlation results of the whole flock and for the trichostrongylid identified responder and non-responder lambs for cumulative weight gain against eosinophil numbers. No significant associations were observed between these two traits in the whole flock or with any of the selected responder and non-responder lambs.

	Trichostrongylid	
	Rho (ρ)	P value
Whole Flock	-0.04	0.72
Responders		
Mid Season	-0.05	0.89
End Season	0.20	0.57
Overall Season	0.46	0.18
Non-Responders		
Mid Season	0.12	0.75
End Season	0.06	0.86
Overall Season	-0.32	0.37

Table 5.10 Correlations of the whole flock and selected trichostrongylid responders and non-responders between cumulative weight gain and eosinophil counts over the last four time points

Graphs were produced of the mean cumulative weight gain against the mean immunological parameters measured (eosinophils and IgE bearing cells) of selected responders and non-responders over the last period of the grazing season. A horizontal line and vertical line were applied to the graphs to illustrate the mean cumulative weight gain and mean immunological factor of the whole flock of lambs, respectively. The lambs were located in different sections of the resultant quadrant. Figure 5.15 shows the four different sections and illustrates how the cumulative weight gain and immunological parameters relate to each other, for example, the top left quadrant demonstrates a increased weight gain and low immunological parameter.

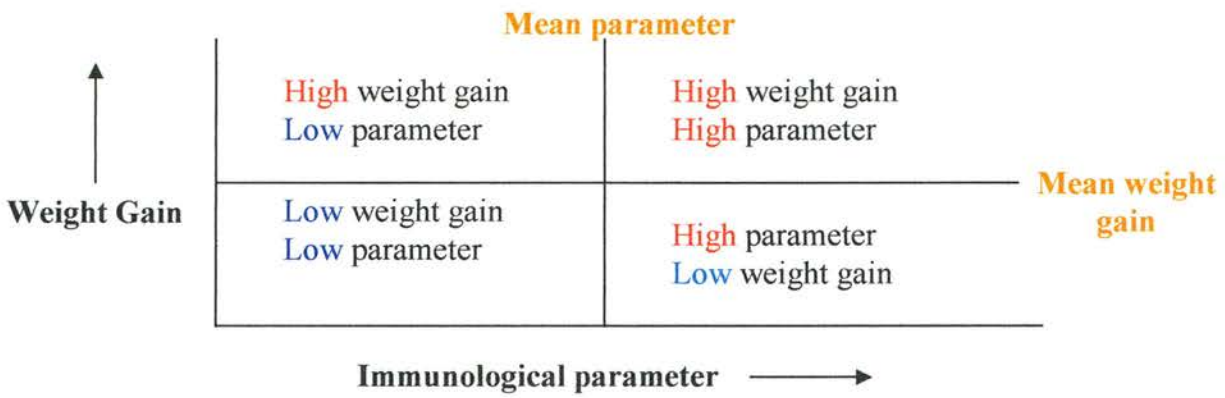


Figure 5.15 Schematic plot of cumulative weight gain against immunological parameters

Figure 5.16 shows a plot of mean cumulative weight gain against mean eosinophil numbers of trichostrongylid selected responders and non-responders over the last four time points of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean eosinophil number and mean cumulative weight gain of the whole flock of lambs, respectively. It was then determined in which quadrant the identified responders and non-responders were located. Table 5.11 demonstrates the % of responders that were found in the high weight gain and high eosinophil count section of the graph and the % of non-responders that were situated in the low weight gain and low eosinophil count part of the graph as indicated by Figure 5.16 including the lambs selected using the trichostrongylid egg counts. In all three graphs (Mid, End and Overall) in Figure 5.16, 50%, 30% and 10% of the responders were distributed in the high cumulative weight gain and high eosinophil section of the

graph, respectively, and 60%, 70% and 80% of the non responders were situated in the low cumulative weight gain and low eosinophil section, respectively.

	Trichostrongylids	
	% R	% NR
Mid Season	50	60
End Season	30	70
Overall Season	10	80

Table 5.11 Percentage of responders and non-responders present in their predicted quadrant

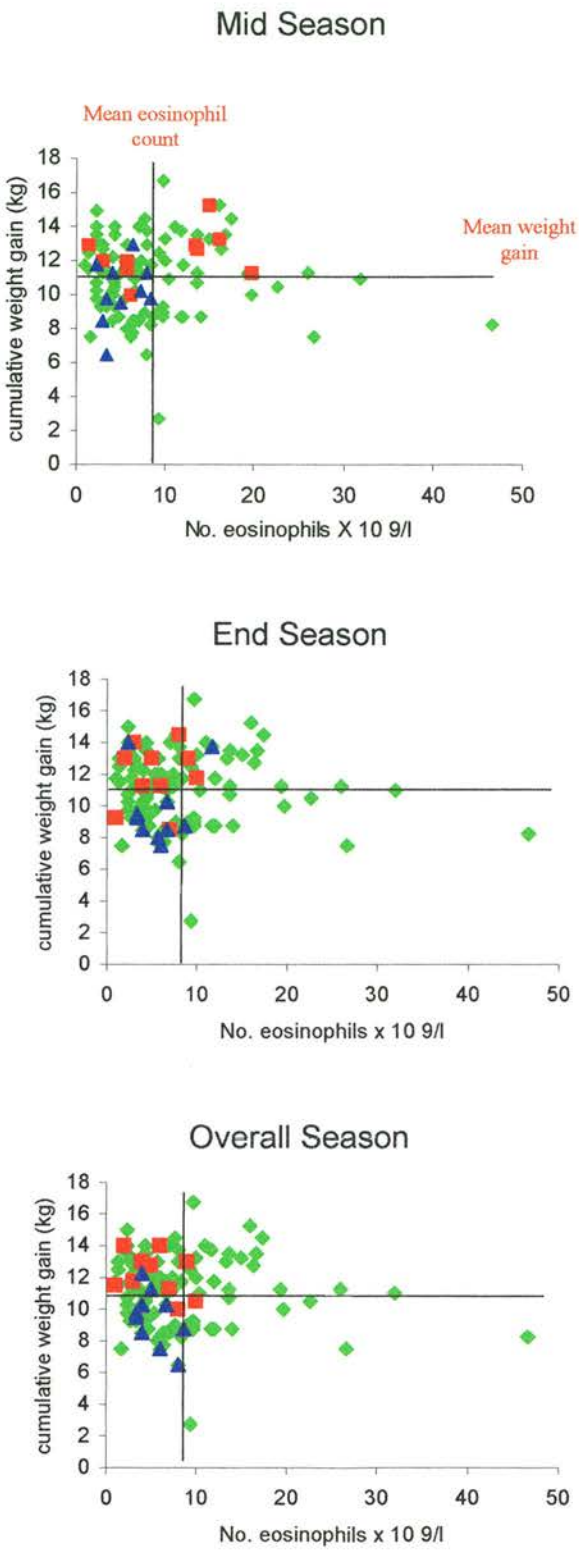


Figure 5.16 Plots showing mean cumulative weight gain vs. mean eosinophil number of trichostrongylid responders and non-responders for the last four time points of the grazing season

5.3.9.2 Cumulative weight gain versus % IgE bearing cells for lambs selected using trichostrongylid *FECs*

Table 5.12 demonstrates the correlation results of the whole flock and for the trichostrongylid identified responder and non-responder lambs for cumulative weight gain against numbers of IgE bearing cells. No significant associations were observed between these two traits in the whole flock or with any of the selected trichostrongylid responder and non-responder lambs.

	Trichostrongylid	
	Rho (ρ)	**P value**
Whole Flock	0.18	0.08
Responders		
Mid Season	-0.11	0.76
End Season	0.54	0.11
Overall Season	0.42	0.23
Non-Responders		
Mid Season	0.19	0.60
End Season	0.25	0.49
Overall Season	-0.47	0.17

Table 5.12 Correlations of the whole flock and selected trichostrongylid responders and non-responders between cumulative weight gain and % IgE bearing cells over the last four time points

Figure 5.17 shows a plot of mean cumulative weight gain against mean % IgE bearing cells in trichostrongylid selected responders and non-responders over the last four time points of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean % IgE bearing cells and mean cumulative weight gain of the whole flock of lambs, respectively. It was then determined in which quadrant the identified responders and non-responders were found. Table 5.13 demonstrates the % of responders that were found in the high weight gain and high IgE bearing cell section of the graph and the % of non-responders that were situated in the low weight gain and low IgE bearing cell part of the graph as indicated by

Figure 5.17 including the lambs selected using the trichostrongylid egg counts. In all three graphs (Mid, End and Overall) in Figure 5.17, 40%, 30% and 10% of the responders were distributed in the high cumulative weight gain and high IgE bearing cell part of the graph, respectively, and 60% of the non responders in each of the three graphs were situated in the low cumulative weight gain and low IgE bearing cell section.

	Trichostrongylids	
	% R	% NR
Mid Season	40	60
End Season	30	60
Overall Season	10	60

Table 5.13 Percentage of responders and non-responders present in their predicted quadrant

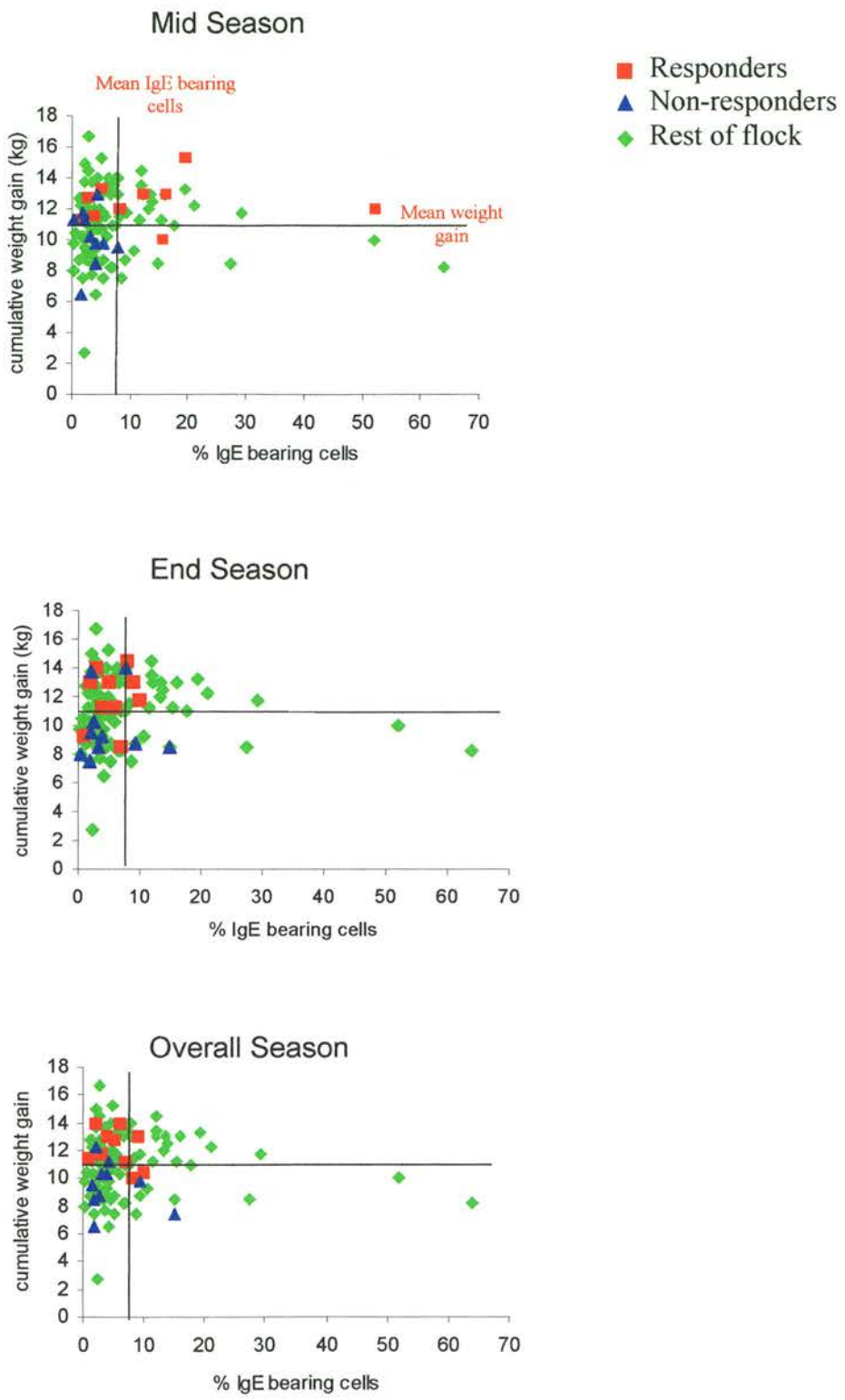


Figure 5.17 Plots showing mean cumulative weight gain vs. mean percentage IgE bearing cells of trichostrongylid selected responders and non-responders for the last four time points of the grazing season

5.4 RESULTS FOR SECOND GRAZING SEASON, 2002

5.4.1 Selected Responder and Non-Responder lambs

The same responder and non-responder lambs selected using the trichostrongylid egg count rankings for the mid, end and overall season (see table 5.1) were followed for a further grazing season.

5.4.2 Lamb faecal egg counts

5.4.2.1 Trichostrongylid *egg counts*

Figure 5.18 displays the FECs of the selected responder and non-responder lambs from the three different time points over the grazing season. The average FEC of the flock was included on each graph. The responder and non-responder lambs selected using the FEC scores from the middle of the season and the overall season followed the same FEC pattern across the season. The egg count levels were elevated at the beginning of the second grazing season in March and then they decreased to very low levels in May. The FECs of both groups subsequently increased to approximately 150epg in July, declining to low levels in August through to October. The animals selected using FEC levels from the end of the season followed the same pattern as the mid and overall season graph from March to May and from August to October, however a different trend was observed in the middle part of the season. The FEC of the whole flock peaked in July and decreased in August as demonstrated in the other two graphs but the FEC of both the responders and non-responders peaked in June and subsequently decreased a month earlier in July.

5.4.2.2 Nematodirus egg counts

Egg counts of *Nematodirus battus* were only observed at the beginning of the second grazing season in the mid, end and overall season graph. From May onwards the *Nematodirus* egg count levels were zero as shown in Figure 5.19.

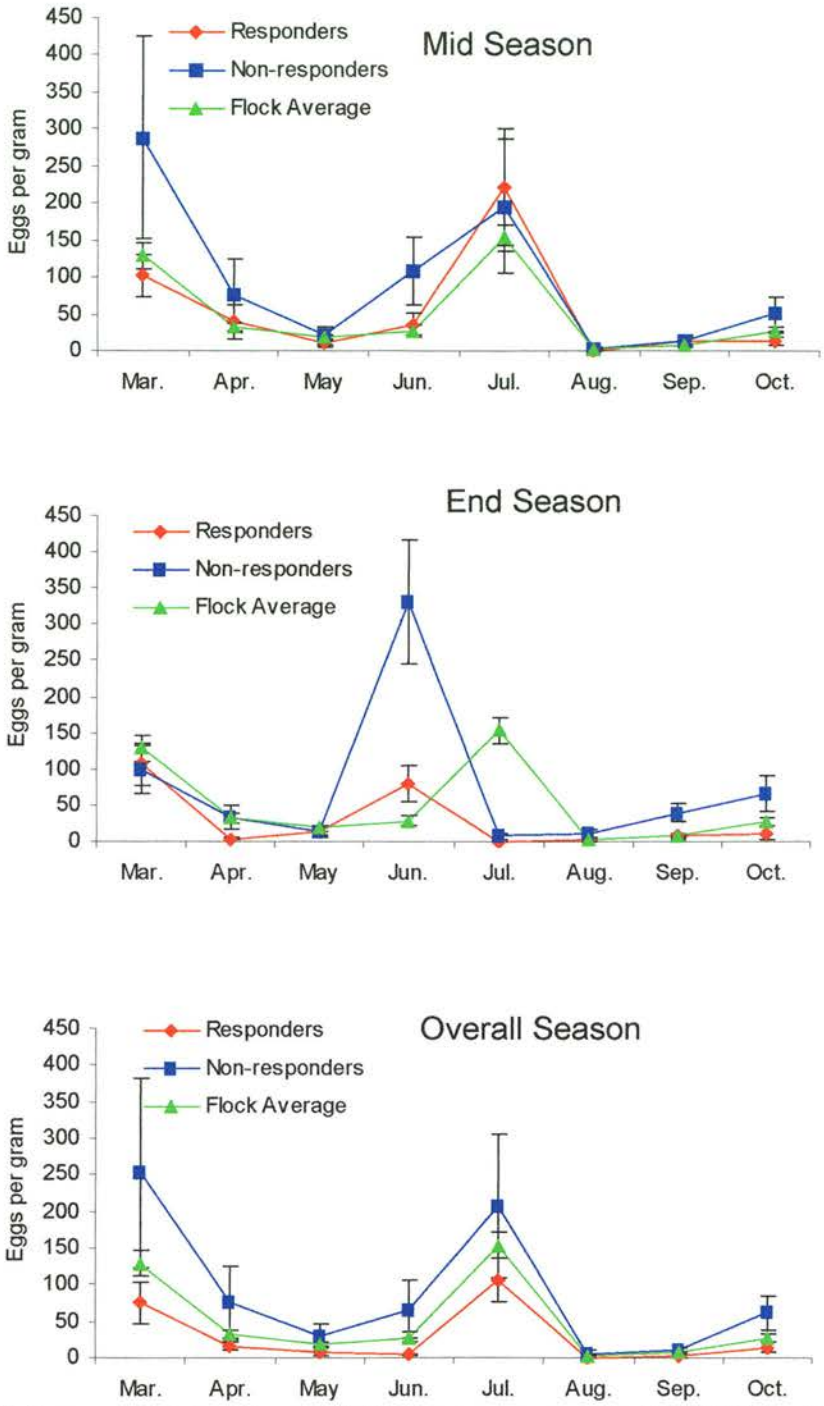


Figure 5.18 Average trichostrongylid faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the second season

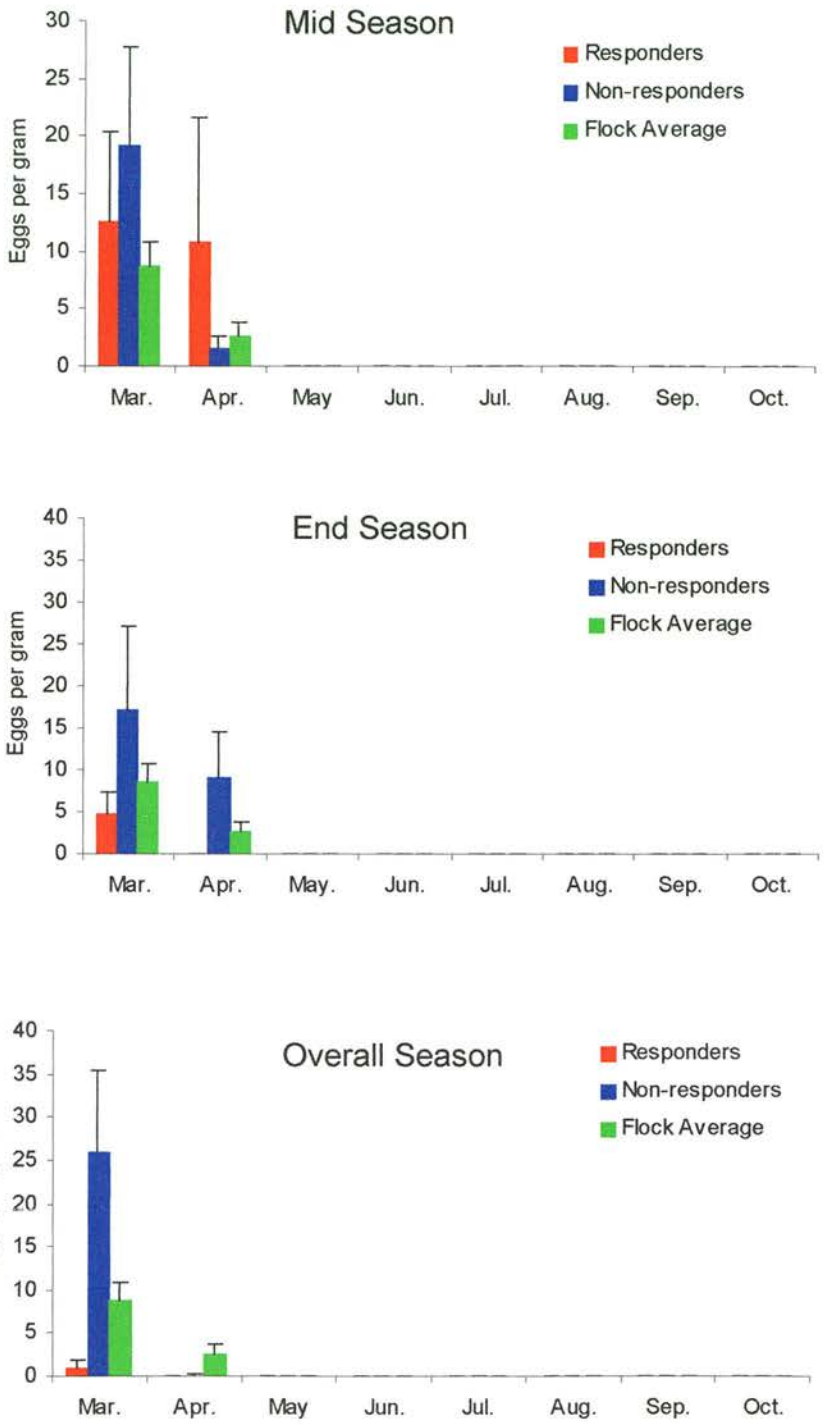


Figure 5.19 Average *Nematodirus* faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points of the second grazing season.

5.4.3 Parasite Specific ELISAs

5.4.3.1 IgE Antibody ELISAs

Figure 5.20 demonstrates the average *T. circumcincta* specific IgE antibody levels for the selected responder and non-responder lambs from the middle, end and overall grazing season. The average IgE antibody concentration of the whole flock was included on each graph. No significant differences in IgE antibody titres were observed between the selected responder and non-responder lambs in all three graphs. In the mid season graph, IgE levels in both groups did not begin to rise until June. IgE levels then gradually increased for two months and subsequently declined from September onwards. The IgE levels of the responders were higher than the non-responders across the grazing season although these differences were not significant. The IgE antibody concentrations in the end and overall season graph followed the same pattern as the mid season graph. However, no differentiation between the responders and non-responders was observed.

5.4.3.2 IgA Antibody ELISAs

Figure 5.21 shows the average parasite specific IgA antibody levels for the selected responder and non-responder lambs from the different time points over the grazing season. A peak in IgA response was seen in the responder group of lambs in all three graphs in April and again in August. The responders were generally higher in IgA levels across the season in the mid, end and overall season graphs. However significant differences in IgA antibody titres between the responders and non-responders were only seen in the end season graph from May to July ($p<0.05$).

5.4.3.3 IgG Antibody ELISAs

The average parasite specific IgG antibody levels for the selected responder and non-responder lambs from the middle, end and overall grazing season were demonstrated in Figure 5.22. The IgG concentration in both groups of lambs in all three graphs remained at a constant level throughout the grazing season. No significant differences in IgG titres were observed between the responders and non-responders at any time point over the season.

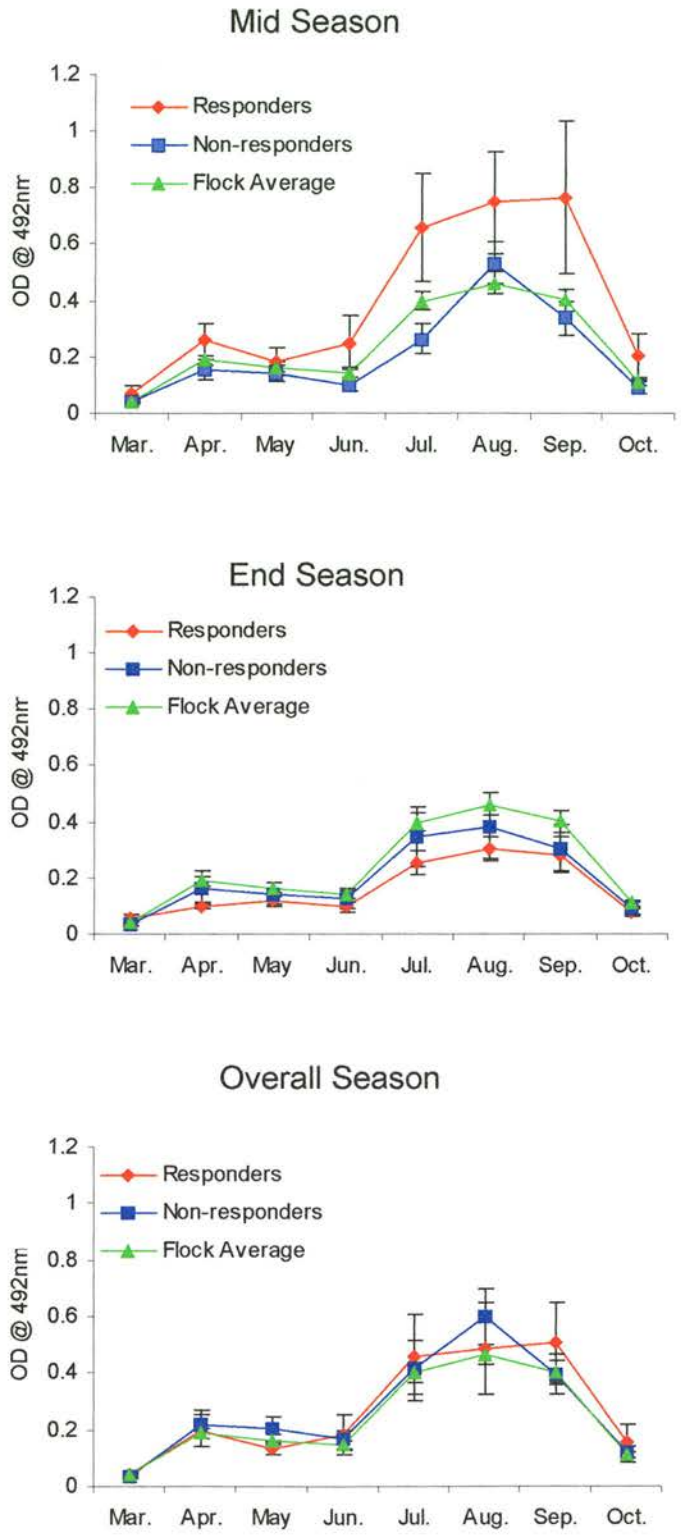


Figure 5.20 Average Parasite Specific IgE Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

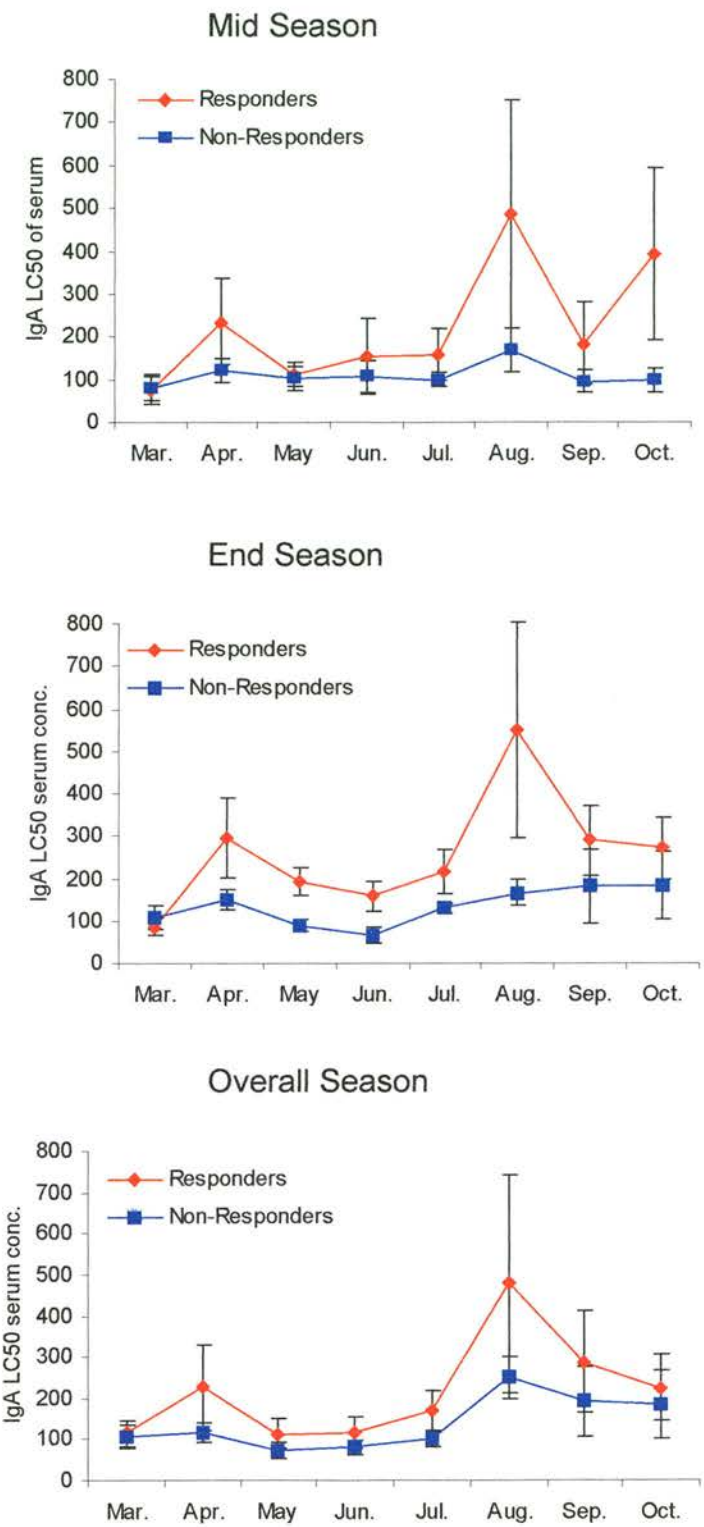


Figure 5.21 Average Parasite Specific IgA Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

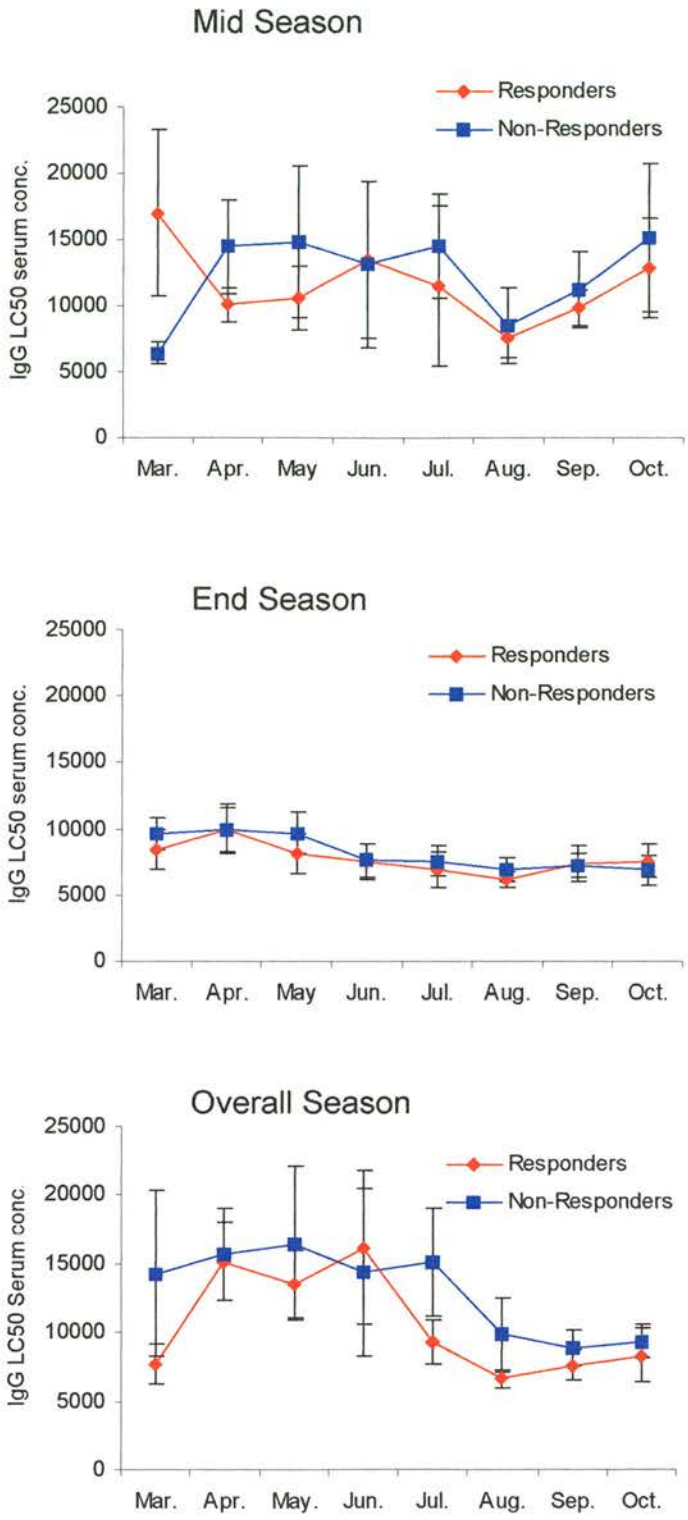


Figure 5.22 Average Parasite Specific IgG Antibody Levels (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

5.4.4 Eosinophil counts

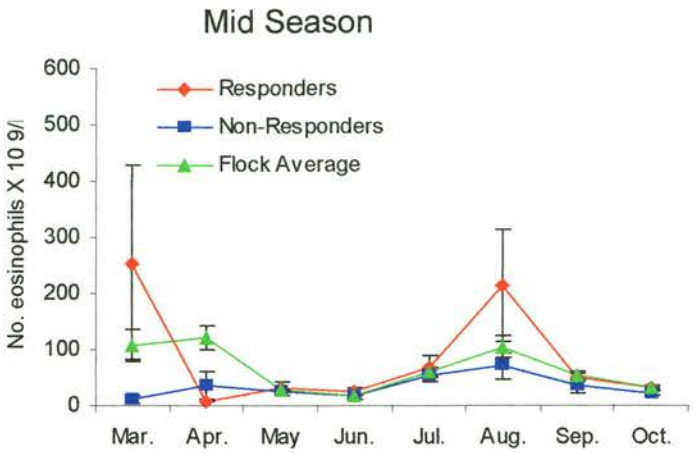
Figure 5.23 shows the average number of eosinophils x 10^9/litre for the selected responder and non-responder lambs from different time points over the second grazing season. The responder lambs in all three graphs displayed high numbers of eosinophils in March which subsequently declined in April. From April through to October eosinophil numbers were relatively low other than a peak in August for the mid season responders. The eosinophil numbers of the non-responder lambs in all three graphs remained at a low level throughout the season. No significant differences in eosinophils were observed between responders and non-responders in the mid, end and overall season graphs.

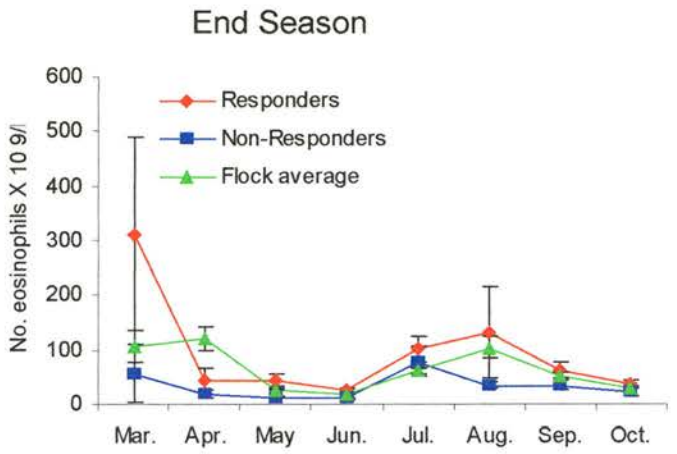
5.4.5 Weights

Figure 5.24 displays the average lamb weight for the selected responder and non-responder lambs from different time points across the second grazing season. In all three graphs, both groups of lambs steadily increased in weight from March to October other than a decrease for all three groups in all three graphs in August. A significant difference in weight between the responders and non-responders was found only in the overall season graph from May to June ($p=0.05$) with the non-responders displaying a heavier weight than the responders.

5.4.6 IgE Bearing Cells

Figure 5.25 shows the average number of IgE bearing cells present for the selected responder and non-responder lambs from different time points over the second grazing season. A trend towards an increase in IgE bearing cells in all animals was observed in the mid, end and overall season graph from March to October. Differences in IgE bearing cells between the responders and non-responders in the end and overall season graph were negligible. However, there were apparent differences in the mid season graph between the responder and non-responder lambs, the former group of lambs exhibiting higher levels of IgE bearing cells. These differences were not significant.





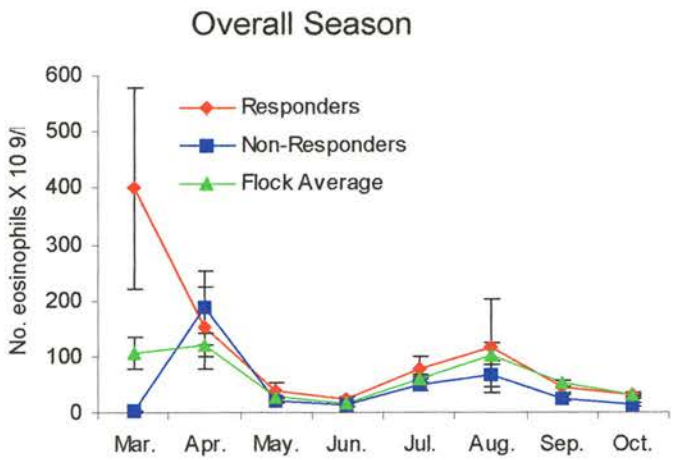


Figure 5.23 Average number of eosinophils x 10^9/l (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

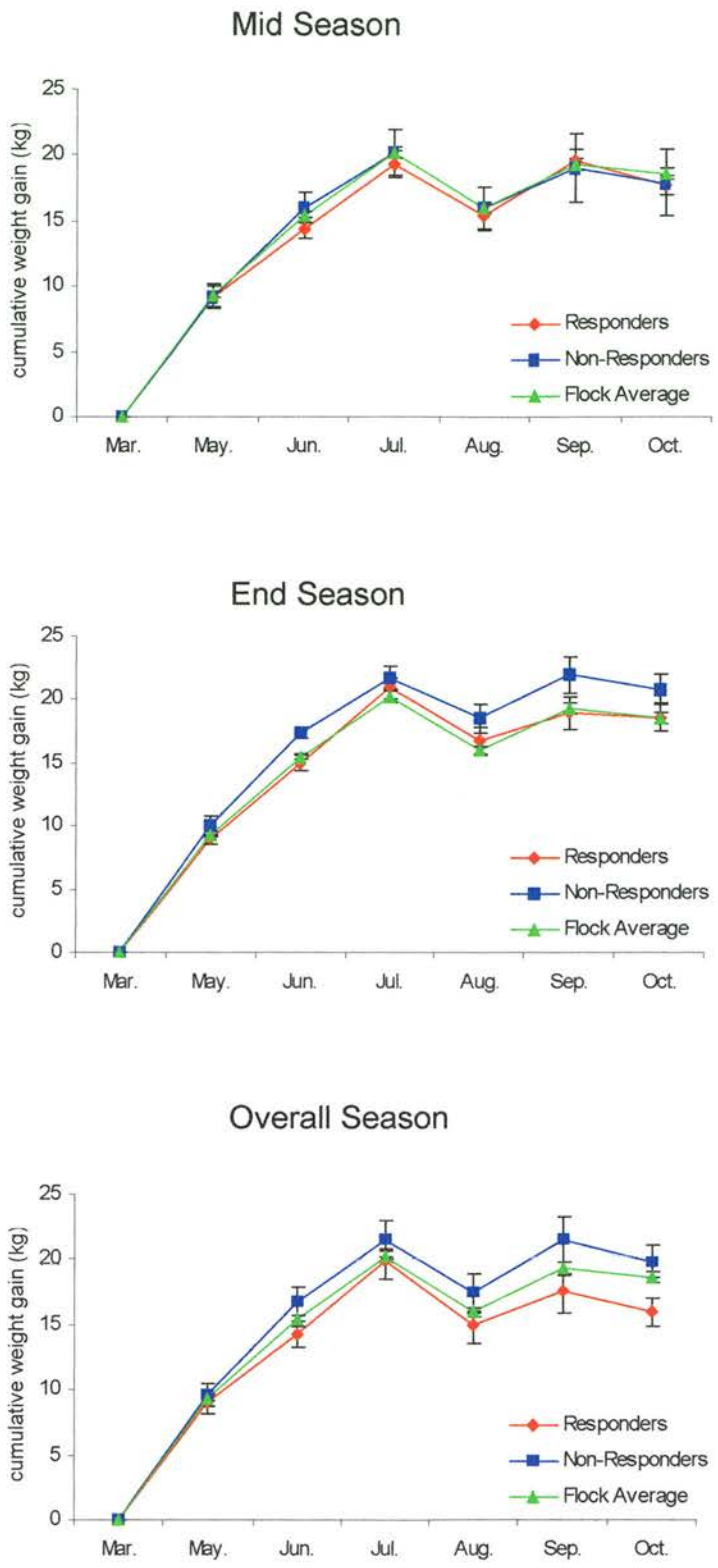


Figure 5.24 Average weight gain (kg) (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

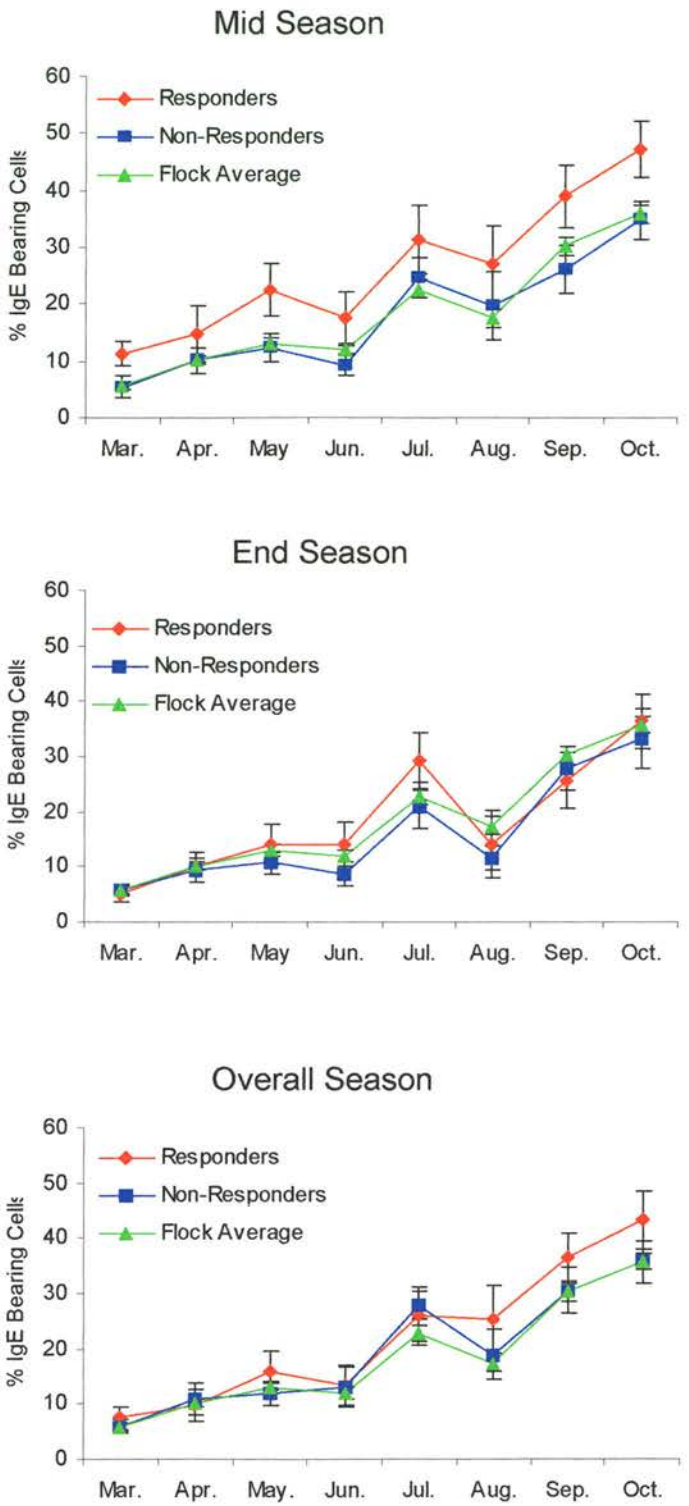


Figure 5.25 Average % IgE Bearing Cells (±SEM) for the selected responder and non-responder lambs from different time points over the second grazing season

5.5 DISCUSSION

Trichostrongylid egg counts have been employed in the previous chapter of this thesis as well as in other sheep and goat studies to segregate animals into "responders" and "non-responders"(Windon, 1990; Woolaston *et al.*, 1992; Baker *et al.*, 1990; Patterson *et al.*, 1996). The results from this chapter are in accordance with the view that the best time to select for responsiveness is when the immunity against the target genera first becomes evident i.e. at the end of the season for trichostrongylids. This reflects the pattern of exposure to the parasites where lambs are exposed to high levels of challenge with trichostrongylid larvae later in the season.

Parasite specific IgE, IgA and IgG antibody levels did not differ significantly between responder and non-responder lambs at any time point during the lambs' first grazing season and overall there was no close association between FEC and parasite specific antibodies. However, samples taken from the whole flock at the end of the first season provided evidence that lambs with higher FEC had lower IgA levels and that responders identified at that time had higher parasite specific IgA levels. These findings are similar to those recorded by Strain *et al.* (2002) that suggests plasma IgA activity may help to select lambs resistant to *T.circumcincta*.

The lack of a strong overall association between peripheral L_3 specific antibody levels and responsiveness focussed attention on the potential use of IgE bearing cells and eosinophils as markers of immunoresponsiveness. A possible benefit in measuring IgE bearing cells is that these cells, although found in the circulation, may migrate to the local lymph nodes where host parasite interactions are mediated. The parasite specific IgE antibodies analysed using the lambs' sera in this study may not be associated directly with the local immune response to nematode infection, and may not provide an adequate index of local antibody production in the abomasal tissues. An additional contrast between these two parameters is that the IgE bearing cells do not measure parasite specific IgE levels, they measure total IgE instead, but studies have shown that the elevation of both parasite specific and total IgE antibody levels are typical features of the sheep's immune response to nematode infection (Shaw *et al.*, 1998). In this study, results showed that towards the end of the season the responders selected using the mid season trichostrongylid egg count rankings had significantly increased IgE bearing cells compared to the non-responders. Additionally, differences in IgE bearing cells between selected end season responders and non-responders were evident but not significant. Also, the plots of mean FEC against mean IgE bearing cells

for these lambs showed that the majority of the end season responder lambs had low FEC and high IgE bearing cells whilst most of the end season non-responders demonstrated high FEC and low numbers of IgE bearing cells. There is therefore the potential for IgE bearing cells to be used as an additional marker for resistance against nematode infections in lambs selected into responders and non-responders using trichostrongylid FECs.

The association between circulating IgE bearing cells and responsiveness in Scottish Blackface lambs is a novel finding. These circulating IgE bearing cells have been demonstrated to be located mainly among lymphocytes and could be classed as B-lymphocytes, circulating systemically prior to progressing to parasite infected mucosae in an approach similar to IgA bearing cells (Hall *et al.*, 1977). The recognition and quantification of these IgE bearing cells may possibly provide a more comprehensive insight into the mechanisms and involvement of systemic IgE antibody. A small quantity of IgE bearing cells was also found among granulocytes in all of the analysed animals. These populations of cells were possibly systemic basophils, which are released into the blood circulation as opposed to mast cells that settle in tissues and usually do not circulate in the blood stream. Moreover, basophils are an important source of effector function in IgE associated immune responses (Kawakami & Galli, 2002). It is also possible that the small population of granulocytes represent circulating eosinophils. Both high and low affinity IgE receptors have been characterised on human eosinophils (Capron *et al.*, 1981) and human eosinophil IgE receptors are involved in IgE mediated immune responses including cytotoxicity against parasites and allergy (Capron *et al.*, 1984). Future studies are therefore required to determine the type of granulocyte involved in bearing IgE antibodies in sheep naturally infected with gastrointestinal parasites.

Blood eosinophilia is a commonly observed characteristic of the sheep's response to nematode infection, with a tendency for selected low FEC sheep lines to demonstrate higher eosinophil counts than high FEC sheep (Buddle *et al.*, 1992; Rothwell *et al.*, 1993; Stevenson *et al.*, 1994). In this study, the identified responder and non-responder lambs using trichostrongylid FECs from the middle and end of the season showed that eosinophil numbers of the responder lambs were significantly higher than the non-responder lambs from late June to early August. The end season trichostrongylid selected responder lambs not only had, as expected, significantly lower trichostrongylid FECs but also had significantly elevated peripheral eosinophil numbers

over the last four time points. Moreover, the end season responder lambs showed a significant association between these two traits, displaying a low FEC and high eosinophil number. A significant correlation was also observed by the mid season non-responder lambs over the last part of the grazing season where the FEC levels were high and eosinophil numbers were low. Analysis of the correlation plots showing mean FEC versus mean peripheral eosinophil count (Figure 5.12) confirms that the majority of the identified end season responders segregated as expected, having below mean FEC coupled with above mean eosinophil counts.

Results suggest so far that it would be advantageous to select responders and non-responders based on late season trichostrongylid FEC levels and mid and end season eosinophil numbers.

The main parasitological parameter used in the breeding of sheep for nematode resistance has been the selection of sheep for a low FEC (Douch *et al.*, 1995). However, studies have demonstrated that under some circumstances the selection of animals for low FEC comes at a cost in terms of production (Williamson *et al.*, 1994). In this study, the weight of the lambs was monitored and the results showed that responder lambs selected using the trichostrongylid egg counts from the middle and end of season gained significantly more weight than the non-responders over the last four time points. No significant correlations were found between FEC and cumulative weight gain in any of the trichostrongylid identified responder and non-responder lambs suggesting that there may be a lack of a strong association between responsiveness and weight gain. However, analysis of correlation plots showing mean FEC versus mean cumulative weight gain (Figure 5.13) demonstrates that the majority of the identified trichostrongylid responder lambs had below mean FEC levels and above mean cumulative weight gain. The majority of the end season non-responder lambs displayed above mean egg output and showed below mean weight gain.

It would appear that responder lambs identified using the trichostrongylid FECs gained significantly more weight than the selected non-responder lambs during the last part of the first grazing season. However, low unfavourable correlations between FEC and production traits were apparent. This may suggest that selecting lambs based on their FEC may affect the productivity of the lambs.

Although it is necessary to find novel immunological markers that can be used in conjunction with other methods to control nematode infections in sheep, it is important to the farmers that selecting for these markers of responsiveness will also not come at a

cost in terms of the animals productivity. Overall, this study has shown that eosinophil levels and IgE bearing cells may have the potential as markers of responsiveness against nematode infection in Scottish Blackface sheep. However, these factors need to be assessed for their effect on productivity of the lambs. The cumulative weight gain of all the lambs was therefore plotted against both eosinophil levels and IgE bearing cells. The results showed that no significant correlations were observed between cumulative weight gain and eosinophil levels within the selected trichostrongylid responder and non-responder lambs during the end of the season. This suggests that there is a lack of strong association between eosinophil levels and productivity.

Within the whole flock of lambs, it was demonstrated that the IgE bearing cells tended to be high when the cumulative weight gain was high, however there was no significant evidence of a strong relationship between these two traits. As a consequence of these results, further studies would need to be employed to determine if selecting for nematode resistance in Scottish Blackface sheep affects the production of lambs.

In this Scottish Blackface study, the pasture was mainly contaminated with *T.circumcincta* and the results demonstrated that the flock of lambs produced on average 1719 million eggs across the whole of the grazing season. The responder lambs selected using the FECs from the middle of the season accounted for 6% of the total pasture contamination and the non-responder group of lambs accounted for approximately 13%. A two-fold difference in egg output was therefore seen between these two groups of lambs selected from the middle of the season. The responder and non-responder lambs selected using the FECs from the end of the season accounted for approximately 4% and 24% of the pasture contamination, respectively resulting in a six-fold difference in egg output between the two groups of lambs. The selected non-responders from the overall season contributed to 19% of total pasture contamination, which was a six-fold increase in egg output than the responder lambs that were only responsible for 3% of the pasture contamination. These results demonstrated that the 10 identified non-responder animals were responsible for approximately one fifth of the pasture contamination, making the animals prime candidates for anthelmintic treatment and in turn, benefiting the farmer in terms of reducing selection pressure for drug resistance.

Although the phenotypic marker used in this study was strongyle faecal egg count the responder animals identified in this way also had lower *Nematodirus* egg counts (see Figure 5.19). Clearly it is useful if animals are equally responsive against both

Nematodirus and the other gastrointestinal trichostrongylids. If this is indeed the case then it may be possible to use *Nematodirus* egg counts as an early season phenotypic marker, this would be valuable for stud farmers who usually sell their ram lambs at the end of the first grazing season.

During the second grazing season, it was evident that parasite specific IgA antibodies were important in the responsiveness of lambs against nematode infection. A significant difference between responders and non-responders was observed during the middle of the season in the group selected on the basis of their end of season trichostrongylid egg counts. Parasite specific IgE and IgG levels were not significant in the lambs' response to nematode infection. Although identified responder lambs demonstrated higher numbers than non-responder lambs of eosinophils and IgE bearing cells across the second grazing season in trichostrongylid mid, end and overall season graphs, the differences were not significant. This was also apparent for the weight gain of the lambs in both groups. It would be interesting for future studies to observe the yearlings' periparturient responses to see what differences occur.

Further controlled studies would be require to gain insight into the between season differences in predominant antibody isotype although it is possible that they simply relate to the different stages in the development and maintenance of immunity in first and second grazing season animals. Lambs during their first grazing season are largely acquiring immunity whereas during their second grazing season they are completing the development and maintainance of these acquired immune responses.

This study has shown that there may be potential for cellular markers and parasite specific antibodies in Scottish Blackface lambs to be used as additional markers to determine how responsive a host is to nematode infection. However, it is important to find an immunological parameter that can be used as an indicator of resistance in other breeds of sheep, not solely Scottish Blackface.

Future research on disease resistance will undoubtedly unravel some of the complex issues relating to within and between-breed differences that have been highlighted both in this study and in the literature to date.

Chapter 6

Characterising responsiveness against *Nematodirus battus* infections in Scottish Blackface Lambs and its relationship with specific immunological and production parameters.

2001

6.1 INTRODUCTION

The life cycle of *N.battus* is different from that of related trichostrongyle nematodes. Most species hatch as L_1 larvae, which develop into L_2 larvae and finally become the infective L_3 larvae which live on pasture. *Nematodirus battus* develops to the L_3 within the egg before hatching (Thomas & Stevens, 1956). Moreover, a rapid increase in *N.battus* on pasture occurs over a short period of time during spring (March to May) in the UK (Thomas & Stevens, 1956) whereas trichostrongyles are usually present on the pasture in much lower numbers at this time of the season.

An ability to accurately identify individual animals bearing desirable traits at the earliest possible stage is clearly important. This is particularly important in the identification of ram lambs with an enhanced ability to respond against gastrointestinal nematodes since these animals are usually sold at the end of their first grazing season. In Britain the first gastro-intestinal nematode that lambs are exposed to in large numbers and more importantly acquire immunity against is *N.battus* (Winter 2002). The aim of this study was to identify responder and non-responder Scottish Blackface lambs on the basis of their *N.battus* faecal egg counts. Comparison between individuals selected using either *Nematodirus* or trichostrongylid egg counts in which *Teladorsagia* was the predominant genera should provide some indication of the sensitivity of the faecal egg count selection process and provide some preliminary indication of how closely linked the immunoregulatory processes are for these two genera.

6.2 MATERIALS AND METHODS

The methods used to determine FEC, eosinophils, IgE bearing cells, and weight gain are those described in chapter 5. Scottish Blackface lambs were ranked in order of their cumulative resistance to nematode infection as determined by their individual *N battus* FECs for the middle of the season (27th Jun-8th August'01) and also the end of the season (7th September-17th October'01). The ten lambs that had the lowest overall egg count were termed "responders" and the animals with the highest egg count were termed "non-responders". The average of the FEC rankings for each of these time periods was then calculated and an overall rank was given for each of the animals.

animals consisted all together of 7 responder lambs and 7 non-responder lambs from the animal numbers selected using the mid season and end season FEC rankings.

Middle Season		End Season		Overall Season	
R	NR	R	NR	R	NR
639	746	699	687	653	746
652	835	740	689	699	779
653	856	744	779	754	783
682	909	835	780	771	883
687	926	850	783	813	916
723	1007	857	861	857	924
813	1013	1022	898	1012	978
900	1070	1043	916	1022	1007
960	1104	1082	924	1071	1013
1012	1144	1099	1007	1099	1015

Table 6.1 Animals selected as responders and non-responders using their *Nematodirus* FEC rankings from the mid, end and overall season

6.3.2 Faecal egg counts of selected responder and non-responder lambs using *Nematodirus* FEC rankings

6.3.2.1 Nematodirus *egg counts*

Figure 6.1 shows the average egg counts of the responder and non-responder lambs selected using the *Nematodirus* FEC rankings from the three different time points over the grazing season. The average FEC of the flock was included on each graph. The *Nematodirus* egg count value of the responder lambs selected from the middle of the grazing period was ~350 epg at the first time point. Following the first anthelmintic treatment however, the egg counts declined to zero and remained low from late June through to late August. The number of eggs then began to increase steadily to levels of ~ 100 epg in late October. The *Nematodirus* FECs of the non-responder lambs from the mid season graph followed a very different pattern subsequent to the first anthelmintic treatment. They increased significantly to egg count levels of ~ 550 epg in late July and then began to decrease prior to the second

6.3.2 Faecal egg counts of selected responder and non-responder lambs using *Nematodirus* FEC rankings

6.3.2.1 Nematodirus *egg counts*

Figure 6.1 shows the average egg counts of the responder and non-responder lambs selected using the *Nematodirus* FEC rankings from the three different time points over the grazing season. The average FEC of the flock was included on each graph. The *Nematodirus* egg count value of the responder lambs selected from the middle of the grazing period was ~350 epg at the first time point. Following the first anthelmintic treatment however, the egg counts declined to zero and remained low from late June through to late August. The number of eggs then began to increase steadily to levels of ~ 100 epg in late October. The *Nematodirus* FECs of the non-responder lambs from the mid season graph followed a very different pattern subsequent to the first anthelmintic treatment. They increased significantly to egg count levels of ~ 550 epg in late July and then began to decrease prior to the second anthelmintic treatment reaching negligible levels in late August. From this time point onwards, they followed the same pattern as the responder lambs. The end season graph showed that the responders and non-responders shared a similar egg count pattern from early June to late August with both groups of lambs peaking in late July with an egg count of ~ 250 epg. Egg count levels then began to decrease prior to the second anthelmintic treatment being administered and reached zero egg count levels in late August. Subsequently, the responder lambs remained at zero, whilst the egg counts of the non-responder lambs increased to ~ 250 epg by late October. The overall season graph followed the same egg count pattern as the mid season graph from early June to late August and for the remainder of the season, similarities in egg counts were observed with the end season graph.

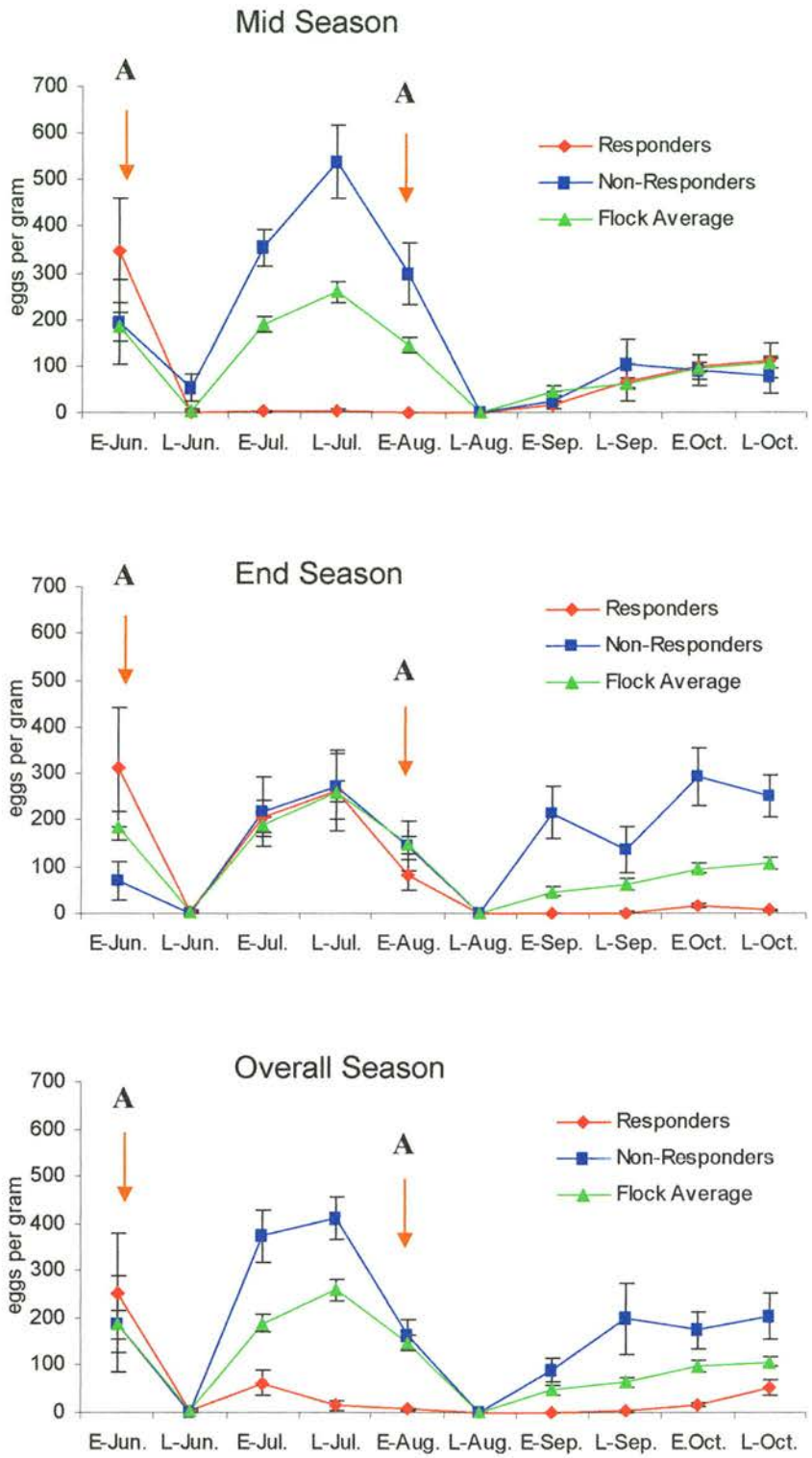


Figure 6.1 Average *Nematodirus* faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using *Nematodirus* FEC rankings

6.3.2.2 Trichostrongylid *egg counts*

Figure 6.2 shows the average trichostrongylid egg count levels of the responder and non-responder lambs selected using the *Nematodirus* FEC rankings from three different time points over the grazing season. The average FEC of the flock was included on each graph. The mid season graph demonstrated that no noticeable differences were observed between the egg count levels of the selected responders and non-responders across the grazing season. The end season graph showed that the responder and non-responder lambs followed the same pattern in egg counts from early June to early July. Following this time point however, the responder lambs displayed a peak in egg count levels at ~ 300 epg in late July, where as the non-responders demonstrated a peak in egg count levels in early August at ~ 350 epg. After the second anthelmintic treatment, the egg count levels of both groups of lambs decreased to zero. The FECs of the responder lambs, subsequently remained at low levels for the rest of the season. The FECs of the non-responders steadily increased to egg count levels of ~ 275 epg. The overall season graph had a similar pattern in FECs in both groups of lambs compared to the end of season graph.

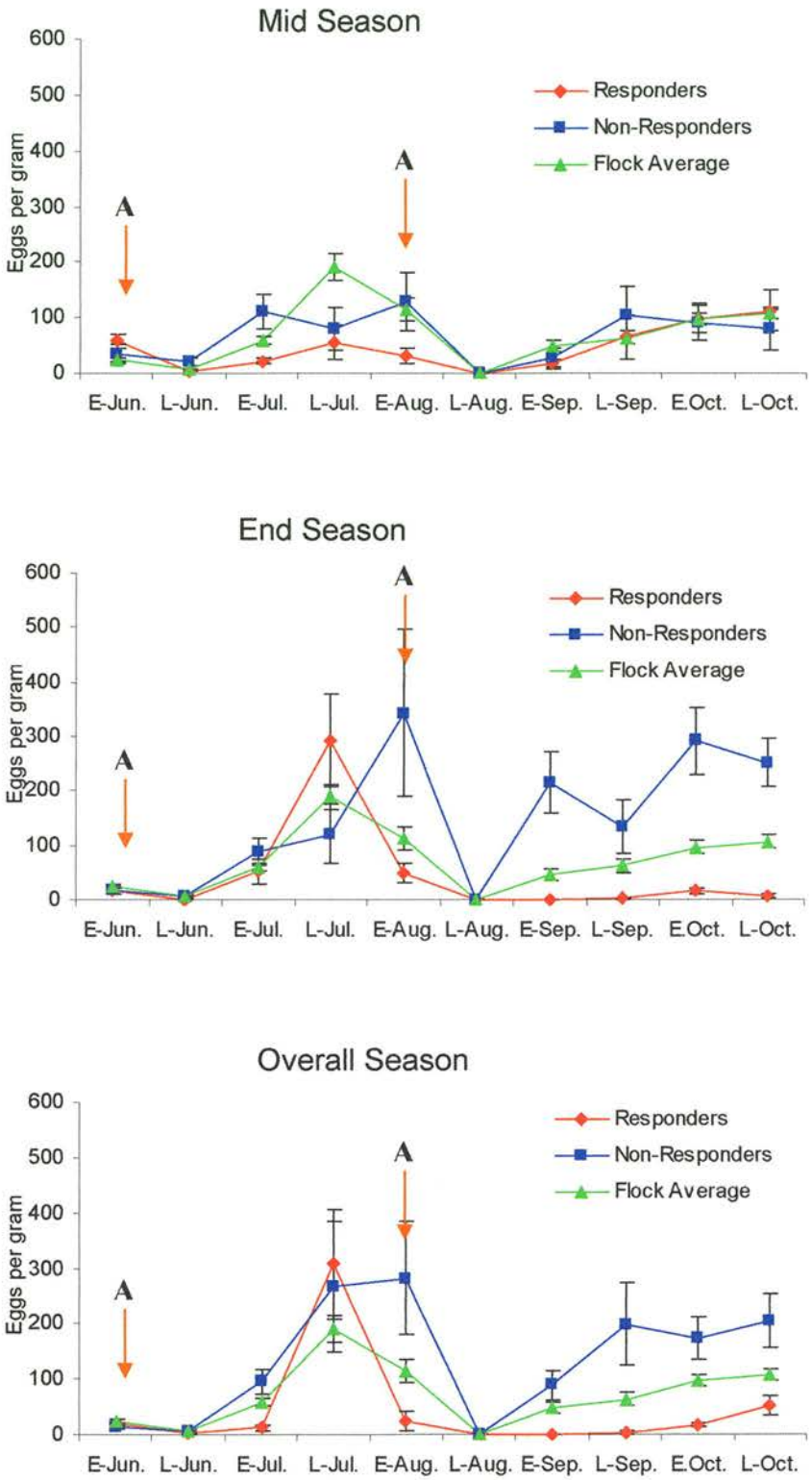


Figure 6.2 Average trichostrongylid faecal egg counts (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using *Nematodirus* FEC counts

6.3.3 Eosinophil counts of *Nematodirus* selected responder and non-responder lambs

Figure 6.3 shows the average number of eosinophils x 10^9/litre for the *Nematodirus* selected responder and non-responder lambs from different time points over the grazing season. Eosinophil numbers in the mid season graph were higher in the responder lambs compared to the non-responder lambs across the grazing season. Moreover, the responder lambs had significantly higher eosinophil numbers than the non-responders during the middle part of the season (Late June to early August) ($p<0.05$). No significant differences in eosinophil concentrations between the selected two groups of lambs were observed in the end season graph. However, higher numbers of eosinophils across the season were observed in the responder group of lambs. Significant differences in eosinophil numbers between responder and non-responder lambs were observed in the overall season graph during the middle and end of the season ($p<0.05$, $p=0.05$, respectively) with the former group displaying higher eosinophil numbers.

6.3.4 Weight gain of *Nematodirus* selected responder and non-responder lambs

The average cumulative weight gain for the *Nematodirus* selected responder and non-responder lambs from the middle, end and overall grazing season is demonstrated in Figure 6.4. No significant difference in the average cumulative weight gain of lambs was observed between the responder and non-responder group of lambs in the mid season graph over any of the time points. The end season graph, however showed that the identified responders gained significantly more weight than the non-responders during the middle part and end of the season ($p<0.01$, $p<0.05$ respectively). The responders selected using the overall season *Nematodirus* FEC rankings gained significantly more weight during the end of the grazing season ($p<0.05$).

6.3.5 IgE bearing cells of *Nematodirus* selected responder and non-responder lambs

The average number of IgE bearing cells for the *Nematodirus* selected responder and non-responder lambs from different time points over the grazing season is

displayed in Figure 6.5. The percentage of IgE bearing cells only began to increase after September in all three graphs. The IgE bearing cells of the responders were marginally higher than the non-responders in October in the mid season graph but these differences were not significant. In the end and overall season graph, the numbers of IgE bearing cells of the responders and non-responders were comparable across the whole of the grazing season.

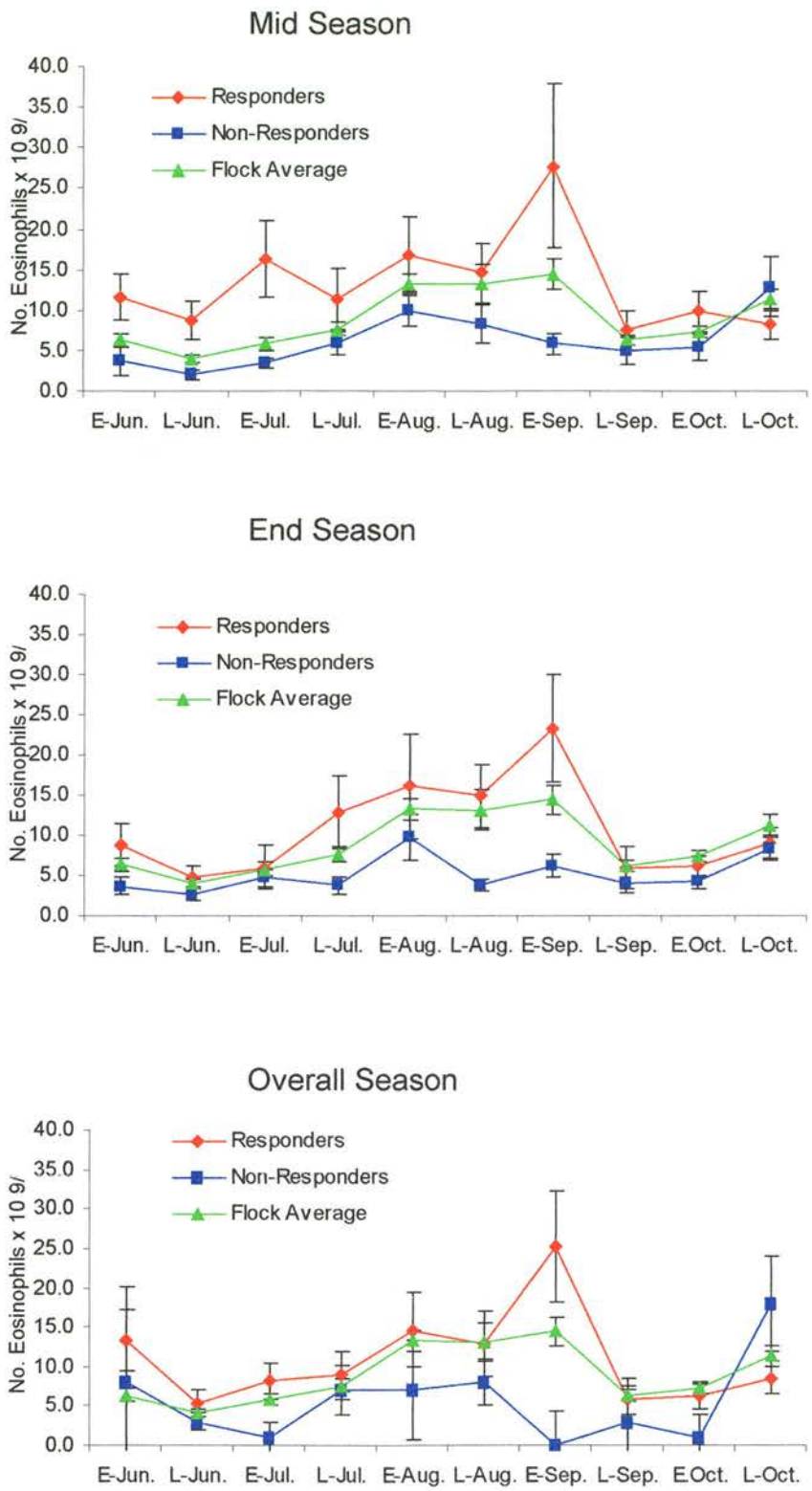


Figure 6.3 Average number of eosinophils x 10^9/l (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using the *Nematodirus* FEC rankings

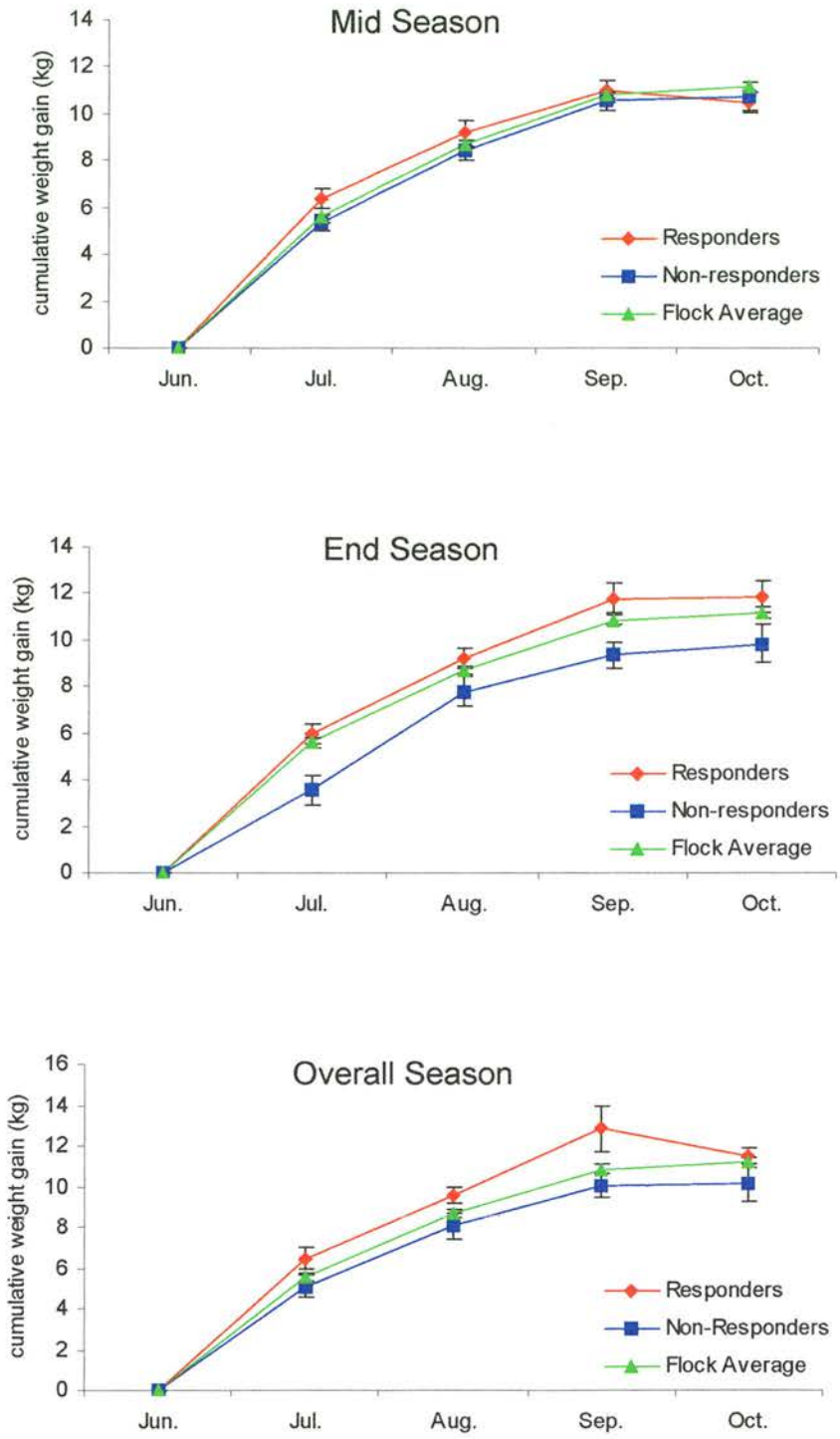
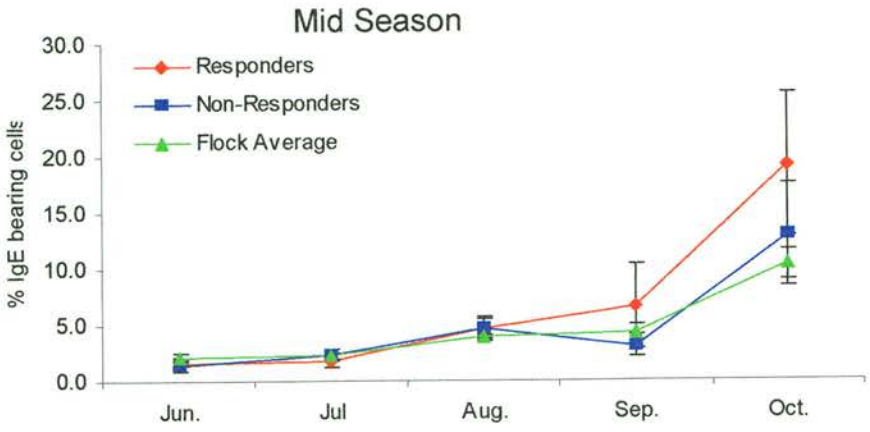
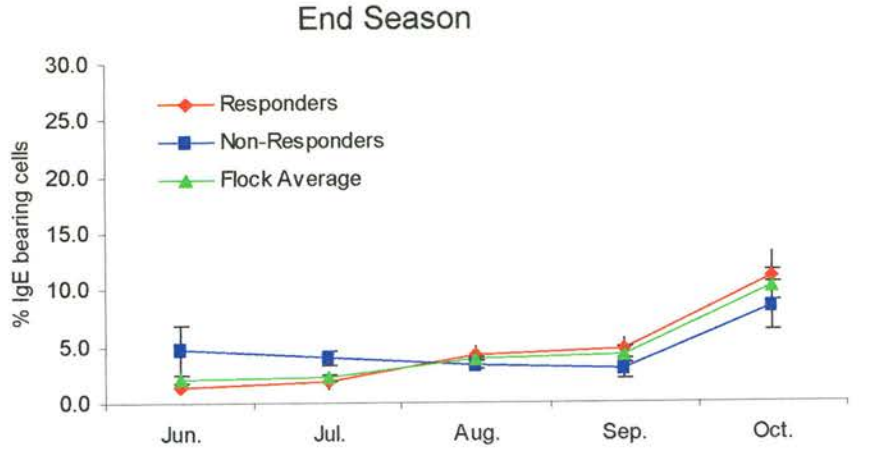


Figure 6.4 Average weight (kg) (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using the *Nematodirus* FEC rankings





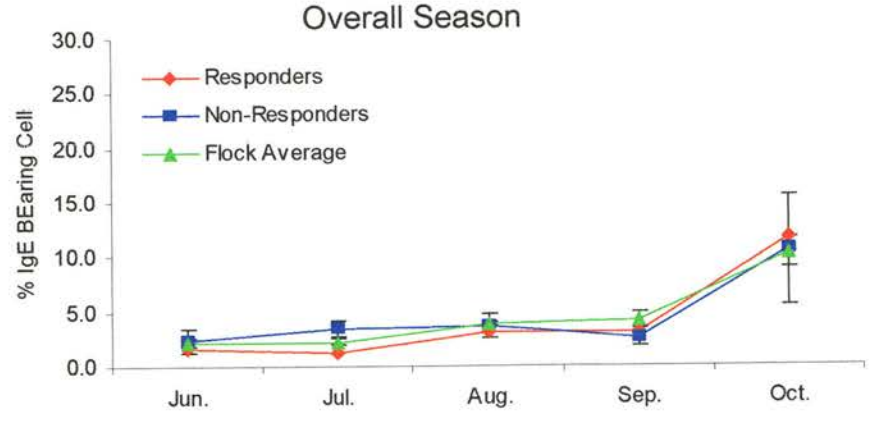


Figure 6.5 Average % IgE Bearing Cells (±SEM) for the selected responder and non-responder lambs from different time points over the grazing season using the *Nematodirus* FEC rankings

6.3.6 *Nematodirus* Correlations

6.3.6.1 Nematodirus *FECS versus eosinophils*

The *Nematodirus* FEC and eosinophil numbers for the whole flock of lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season. The eosinophil numbers of the responders and non-responders selected using the *Nematodirus* FEC rankings from the middle, end and overall part of the season were also analysed over the same time period to establish if they also shared any association with their FECs. Table 6.2 displays the correlation results between *Nematodirus* FEC and the numbers of eosinophils x 10^9/litre. No correlations were observed between FECs and eosinophil numbers in the whole flock. Identified mid season responder and non-responder lambs showed no association between FEC levels and eosinophil numbers. Identified end season responders showed that when FECs were low the eosinophil numbers tended to be high, and a significant linear relationship between these two parameters was observed (ρ= -0.78, p=0.01). The non-responder lambs showed no correlation between these two traits. The selected overall season responder and non-responder lambs showed no strong association between FEC and eosinophil numbers over the last part of the grazing season.

	Rho (ρ)	P value
Whole Flock	-0.11	0.29
Responders		
Mid Season	-0.13	0.73
End Season	-0.78	0.01*
Overall Season	-0.21	0.56
Non-Responders		
Mid Season	-0.02	0.96
End Season	-0.08	0.83
Overall Season	0.13	0.73

Table 6.2 Correlations of the whole flock and selected responders and non-responders between *Nematodirus* FEC and eosinophil numbers over the last four time points

Figure 6.6 shows the plot of mean *Nematodirus* FECs against mean eosinophil numbers of identified responder and non-responder lambs towards the end of the grazing season. The vertical line and horizontal lines on the graphs represent the mean *Nematodirus* FEC and mean eosinophil counts of the whole flock, respectively. All of the graphs in Figure 6.6 (Mid, End and Overall) showed that 40%, 50% and 40% of the responders were found in the low FEC and high eosinophil count section of the graph, respectively and 30%, 90% and 80% of the non-responders were distributed in the high FEC and low eosinophil count section, respectively. In the end season graph, 50% of the responders and 90% of the non-responders were present in their predicted position in the graph.

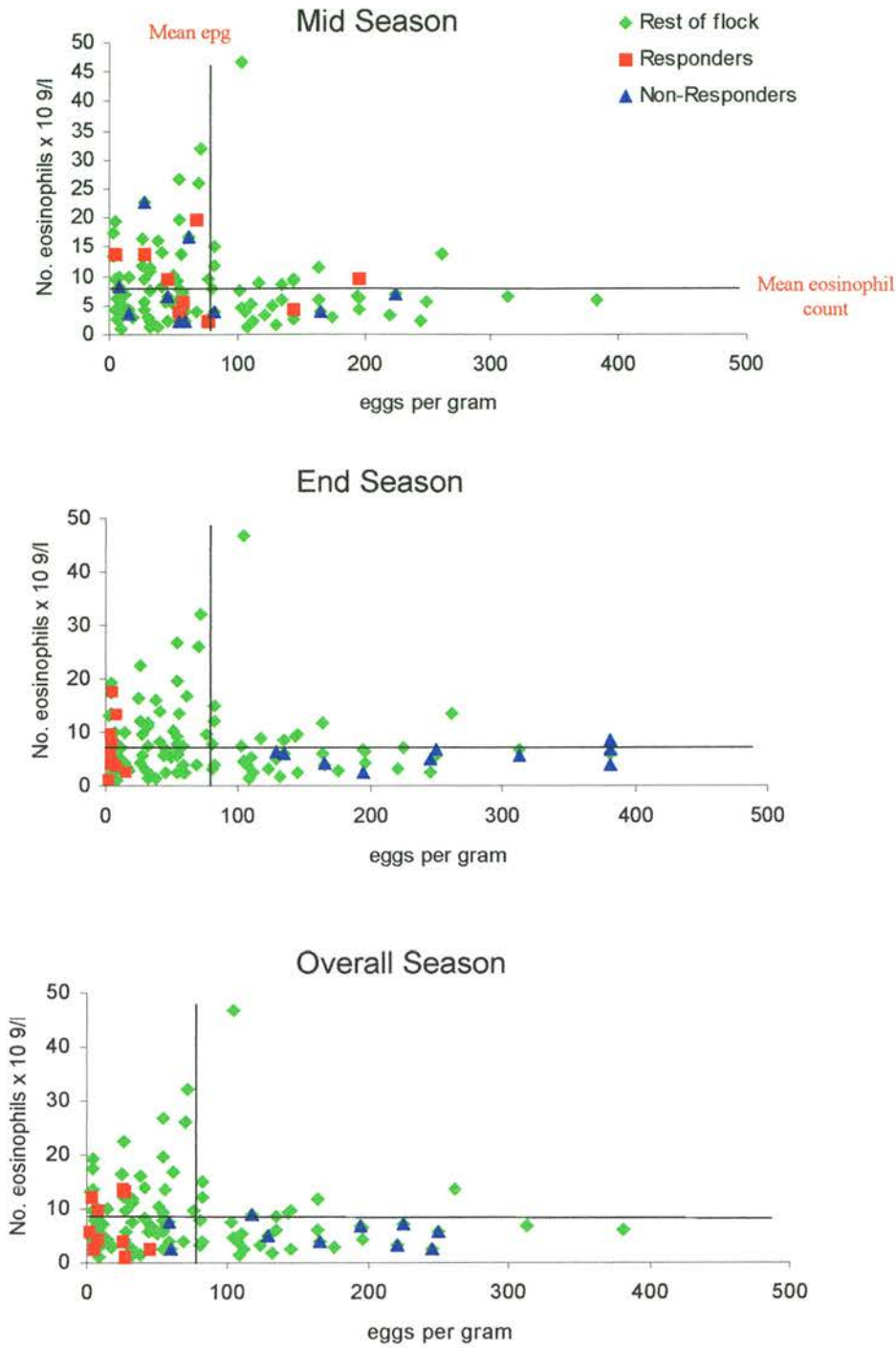


Figure 6.6 Plots showing mean *Nematodirus* FEC vs. mean eosinophil counts of selected responders and non-responders for the last four time points of the grazing season

6.3.6.2 *Nematodirus* FECs versus cumulative weight gain

The *Nematodirus* FEC and cumulative weight gain for the lamb flock and for the identified responder and non-responder lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season. Table 6.3 shows these correlation results. Towards the end of the season, within the flock as a whole, animals tended to gain more weight when FEC levels were low and there was significant evidence of a strong linear relationship between these two traits (ρ= -0.38, p=0.001). The identified mid season responders showed that weight gain in the lambs tended to be elevated when FEC levels were low (ρ= -0.58, p=0.08), however, there was no significant evidence of a strong association between these two parameters. The selected mid season non-responders showed that *Nematodirus* FEC levels tended to be high when the cumulative weight gain was low and there was significant evidence of a linear relationship between these two variables (ρ= -0.64, p=.05). The identified end and overall season responder and non-responder lambs showed no association between the two traits.

	Rho (ρ)	P value
Whole Flock	-0.38	0.001*
Responders		
Mid Season	-0.58	0.08
End Season	-0.50	0.14
Overall Season	0.26	0.48
Non-Responders		
Mid Season	-0.64	0.05*
End Season	-0.42	0.23
Overall Season	-0.34	0.34

Table 6.3 Correlations of the whole flock and selected responders and non-responders between *Nematodirus* FECs and cumulative weight gain over the last four time points

Figure 6.7 shows the plot of mean *Nematodirus* FECs against mean cumulative weight gain of identified responder and non-responder lambs towards the end of the grazing season. The vertical and horizontal lines on the graphs represent the mean *Nematodirus* FEC and mean cumulative weight gain of the whole flock, respectively. The graphs in Figure 6.7 (Mid, End and Overall) show that 50%, 70% and 80% of the responders respectively were located in the low FEC and high weight gain section of the graph. For the non responders (Mid, End and Overall) 20%, 80% and 60% respectively were situated in the high FEC and low weight gain part of the graph.

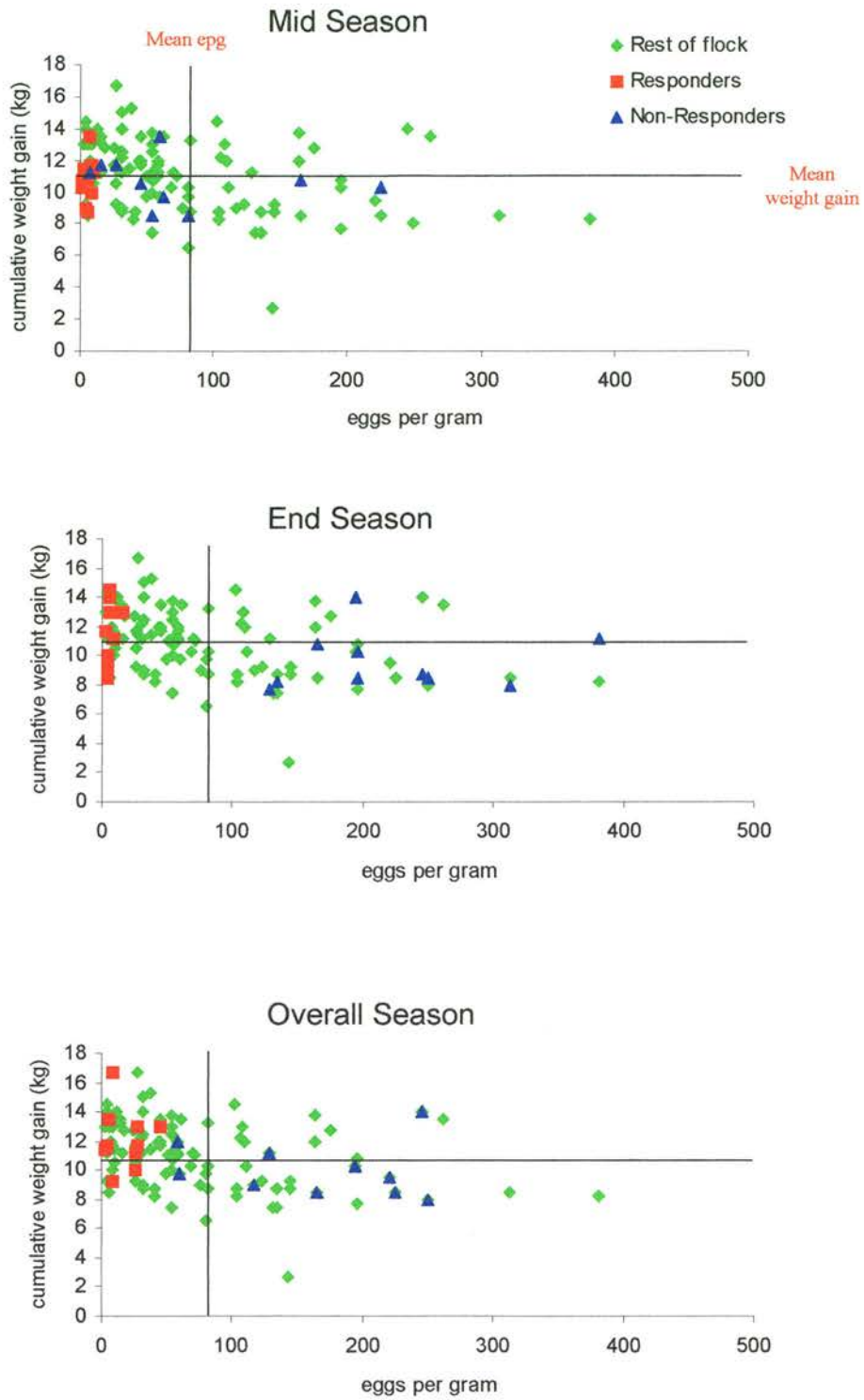


Figure 6.7 Plots showing mean *Nematodirus* FEC vs. mean cumulative weight gain of selected responders and non-responders for the last four time points of the grazing season

6.3.6.3 *Nematodirus FECs versus IgE bearing cells*

The *Nematodirus* FECs and numbers of IgE bearing cells for the lamb flock and for the identified responder and non-responder lambs were analysed to determine if any correlations existed between these traits over the last four time points of the grazing season. Table 6.4 displays these correlation results. Within the whole flock during the end of the season, no correlations between these two traits were observed. The identified mid season responders showed a significant correlation between FEC and the number of IgE bearing cells (ρ= -0.66, $p<0.05$) with the egg output tending to be low and the numbers of IgE bearing cells displaying high values. The selected mid season non-responder lambs plus the identified end and overall season responder and non-responder lambs showed no association between the two traits.

	Rho (ρ)	P value
Whole Flock	-0.17	0.10
Responders		
Mid Season	-0.66	0.04*
End Season	-0.41	0.24
Overall Season	-0.37	0.29
Non-Responders		
Mid Season	0.25	0.49
End Season	-0.16	0.66
Overall Season	-0.02	0.96

Table 6.4 Correlations of the whole flock and selected responders and non-responders between *Nematodirus* FECs and % IgE bearing cells over the last four time points

Figure 6.8 shows the plot of mean *Nematodirus* FECs against mean percentage of IgE bearing cells of identified responder and non-responder lambs towards the end of the grazing season. The vertical and horizontal lines on the graph represent the mean *Nematodirus* FEC and mean percentage of IgE bearing cells of the whole flock respectively. Figure 6.8 shows that 50% (Mid), 60% (End) and 60% (Overall) of the

responders were found in the low FEC and high IgE bearing cell part of the graph. For the non-responders 20%(Mid), 60%(End) and 50% (Overall) were situated in the high FEC and low IgE bearing cell section of the graph.

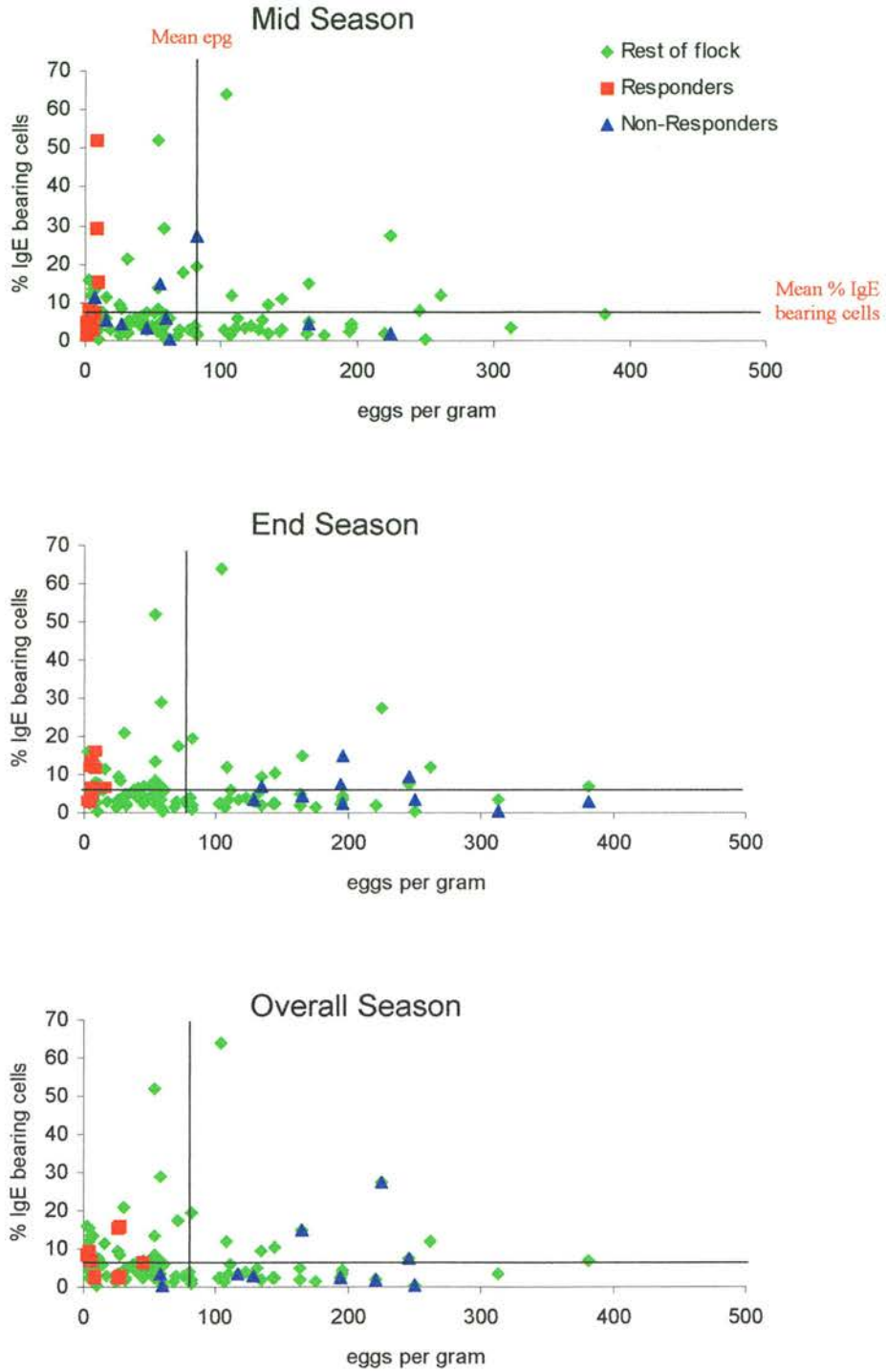


Figure 6.8 Plots showing mean *Nematodirus* FEC vs. mean % IgE bearing cells of selected responders and non-responders for the last four time points of the grazing season

6.3.7 Cumulative weight gain versus immunological parameters for responders and non-responders selected using *Nematodirus* FEC rankings

Correlations were evaluated for cumulative weight gain against eosinophil numbers, and IgE bearing cells in the *Nematodirus* selected responders and non-responders.

6.3.7.1 Cumulative weight gain versus eosinophils for lambs selected using Nematodirus FECs

Table 6.5 demonstrates the correlation results of the whole flock and for the *Nematodirus* identified responder and non-responder lambs for cumulative weight gain against eosinophil numbers. No significant associations were observed between these two traits in the whole flock or with any of the selected responder and non-responder lambs.

	Nematodirus	
	Rho (ρ)	**P value**
Whole Flock	-0.04	0.72
Responders		
Mid Season	-0.57	0.10
End Season	0.32	0.37
Overall Season	0.06	0.87
Non-Responders		
Mid Season	0.33	0.35
End Season	0.28	0.44
Overall Season	0.05	0.89

Table 6.5 Correlations of the whole flock and selected *Nematodirus* responders and non-responders between cumulative weight gain and eosinophil levels over the last four time points

Figure 6.9 shows a plot of mean cumulative weight gain against mean eosinophil numbers of *Nematodirus* selected responders and non-responders over the last four time points of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean eosinophil number and mean cumulative weight gain of the whole flock of lambs, respectively. It was then determined in which quadrant the identified responders and non-responders were located. Table 6.6 demonstrates the % of responders that were found in the high weight gain and high eosinophil count section of the graph and the % of non-responders that were situated in the low weight gain and low eosinophil count part of the graph as indicated by Figure 6.9 including the lambs selected using the *Nematodirus* egg counts.

	Nematodirus	
	%R	**%NR**
Mid Season	20	50
End Season	10	80
Overall Season	40	70

Table 6.6 Percentage of responders and non-responders present in their predicted quadrant

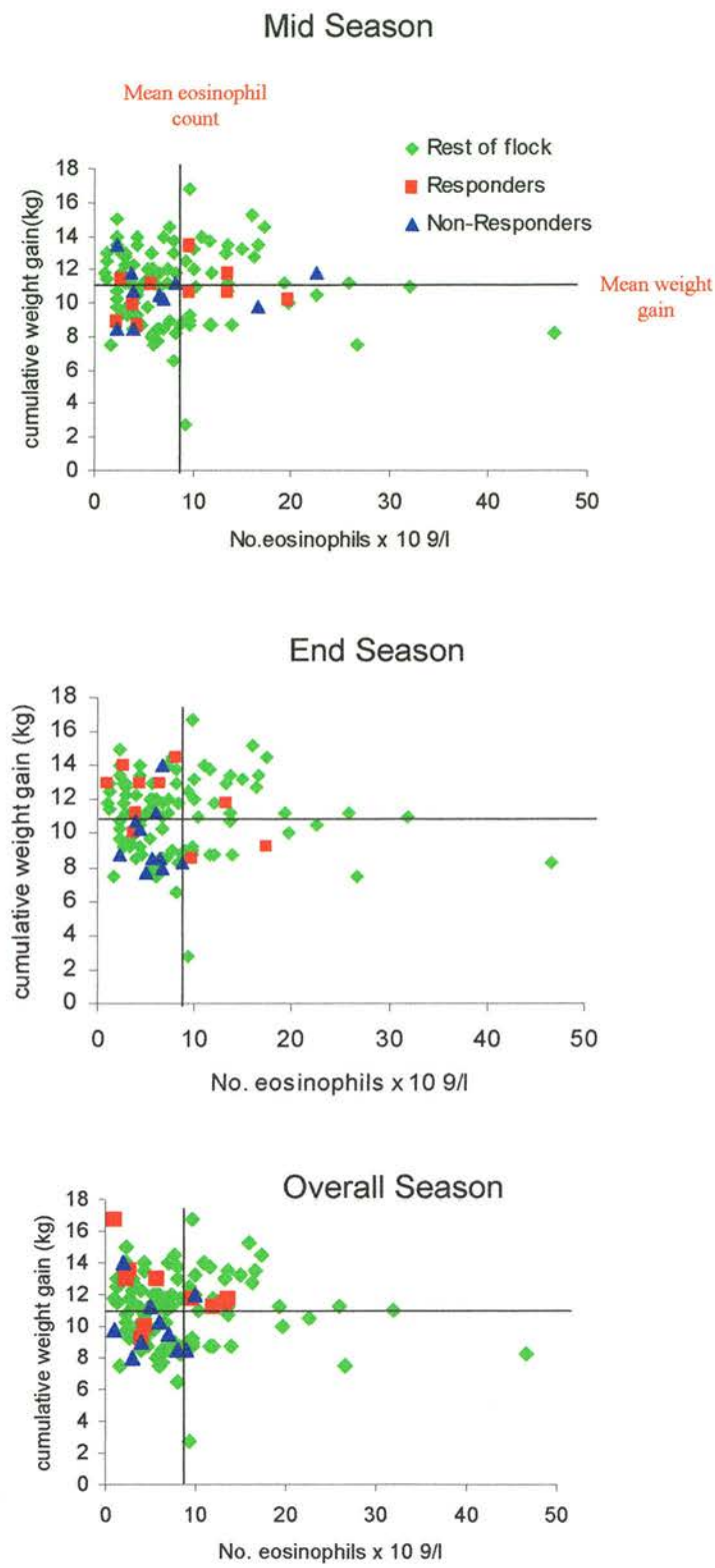


Figure 6.9 Plots showing mean cumulative weight gain vs. mean eosinophil number of *Nematodirus* selected responders and non-responders for the last four time points of the grazing season

6.3.7.2 Cumulative weight gain versus % IgE bearing cells for lambs selected using Nematodirus FECs

Table 6.7 demonstrates the correlation results of the whole flock and for the *Nematodirus* identified responder and non-responder lambs for cumulative weight gain against numbers of IgE bearing cells. There was evidence of a significant association between these two traits in the end of season *Nematodirus* selected responder lambs. The weight gain of these lambs was elevated when IgE bearing cell numbers were high (ρ=0.63, p=0.05). No correlations between these two traits were observed in the *Nematodirus* end of season non-responder lambs or the mid and overall season *Nematodirus* identified responder and non-responder lambs.

	Nematodirus	
	Rho (ρ)	**P value**
Whole Flock	0.18	0.08
Responders		
Mid Season	0.51	0.13
End Season	0.63	0.05*
Overall Season	0.23	0.52
Non-Responders		
Mid Season	-0.06	0.88
End Season	0.14	0.70
Overall Season	0.10	0.79

Table 6.7 Correlations of the whole flock and selected *Nematodirus* responders and non-responders between cumulative weight gain and % IgE bearing cells over the last four time points

Figure 6.10 shows a plot of mean cumulative weight gain against mean percentage IgE bearing cells in *Nematodirus* selected responders and non-responders over the last four time points of the grazing season. A vertical line and horizontal line were added to the graphs to show the mean percentage IgE bearing cells and mean cumulative weight gain of the whole flock of lambs, respectively. It was then determined in which quadrant the identified responders and non-responders were

four time points of the grazing season. A vertical line and horizontal line were applied to the graphs to illustrate the mean % IgE bearing cells and mean cumulative weight gain of the whole flock of lambs, respectively. It was then determined in which quadrant the identified responders and non-responders were found. Table 6.8 demonstrates the % of responders that were found in the high weight gain and high IgE bearing cell section of the graph and the % of non-responders that were situated in the low weight gain and low IgE bearing cell part of the graph as indicated by Figure 6.10 including the lambs selected using the trichostrongylid egg counts and *Nematodirus* egg counts

	Nematodirus	
	%R	**%NR**
Mid Season	0	50
End Season	30	50
Overall Season	40	50

Table 6.8 Percentage of responders and non-responders present in their predicted quadrant

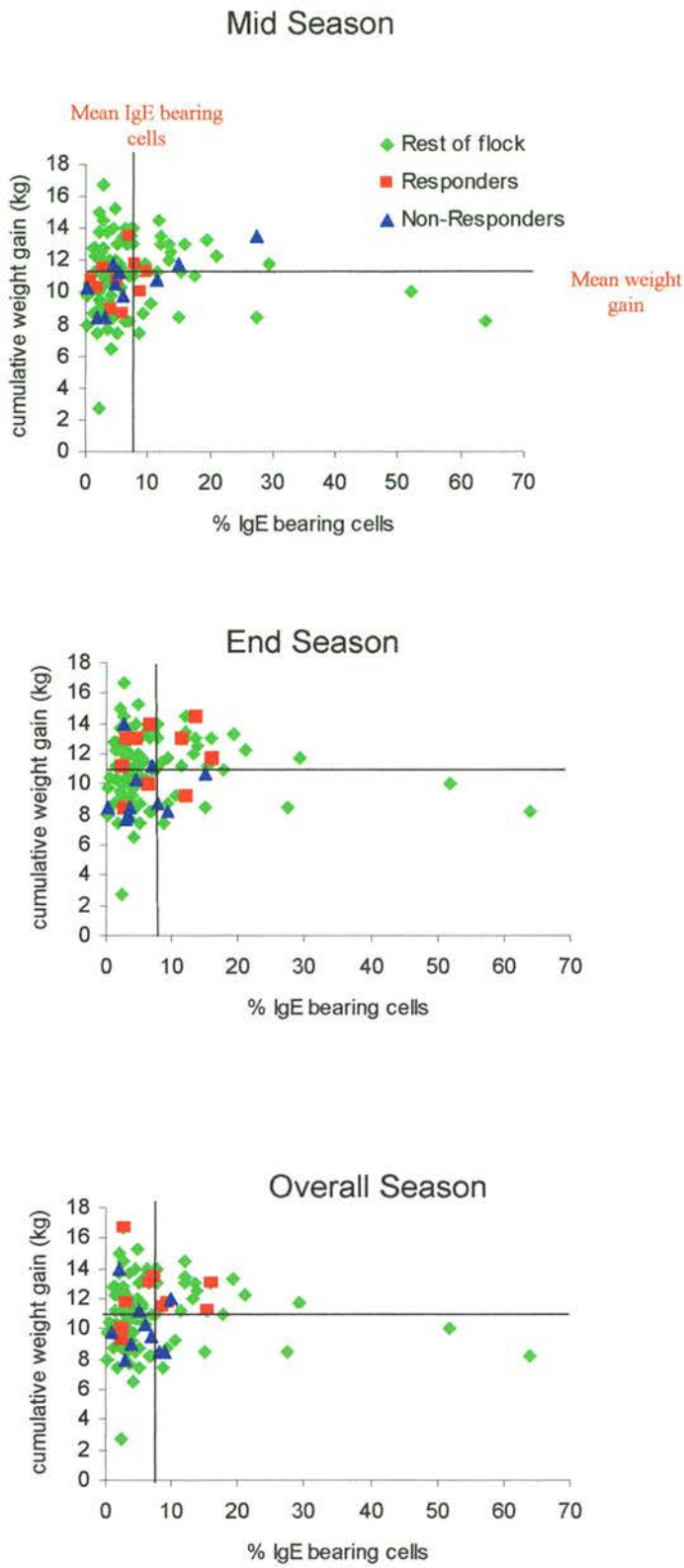


Figure 6.10 Plots showing mean cumulative weight gain vs. mean % IgE bearing cells of *Nematodirus* selected responders and non-responders for the last four time points of the grazing season

6.4 DISCUSSION

The study firstly shows that it is feasible to identify responder and non-responder lambs using *N.battus* FECs. The optimum period for identifying responder and non-responder lambs using *N.battus* egg counts appeared to be in the middle of the grazing season, selection at this time identifying responsive animals that were able to maintain very low *Nematodirus* egg counts throughout the mid summer period and low egg counts in the autumn. Studies on the population dynamics of GI nematodes have shown that there are three phases in the development of immunity, an initial susceptible phase, an acquisition phase and a final phase in which immunity is fully expressed. Previous studies using trichostrongylid FECs (Stear et al 1995a) and Chapter 5 have shown that the optimum time for selecting responsive animals is during the acquisition phase. The fact that it was possible to identify lambs that were responsive against *Nematodirus* relatively early in the grazing season is entirely consistent with previous epidemiological and immunological studies (Winter 2002) which have shown that immunity against this species is first acquired at an early age. However as Figure 6.2 shows those responder lambs selected mid season using *Nematodirus* egg counts did not also have low trichostrongylid egg counts. This finding has some practical implications since it suggest that individuals selected on the basis of being able to rapidly develop effective immunoregulation of Nematodirus egg output would not be able to effectively regulate trichostrongylid egg output. Although the reasons for this are not clear one possible explanation could be that the mechanism(s) regulating egg output for *Nematodirus* and trichostrongylid nematodes may not, at least during the initial phase in the acquisition of immunity against *N.battus*, have a common basis. However those lambs that took longer to acquire an ability to respond against *Nematodirus* i.e. responder lambs selected using late season *Nematodirus* egg counts also appeared to be capable of regulating their trichostrongylid species faecal egg counts. Although this dual responsiveness in these lambs might be explained by a common ovoregulatory effector mechanism it might equally well be attributable to discrete mechanisms. At present we have a poor understanding of the immunoregulatory processes directed against GI nematodes. There is some parasitological evidence derived from studies on periparturient ewes supporting the view that immunoregulation of *N.battus* and trichostrongylid nematodes may be attributable to

mechanisms that differ in ease of maintenance. Despite being subject to considerable challenge with *N.battus* during the periparturient period most ewes are capable of maintaining their immunity against this species thoughout this period. However immunity against many species of trichostrongylid nematodes appears to be more labile and usually wanes during the periparturient period. This relaxation in immunity (periparturient relaxation in immunity [PPRI]) is now thought to arise as a direct consequence of nutritional stress and a re-prioritisation of protein resources duraing late pregnancy and early lactation (Coop & Kyriazakis 2001).

The studies using specific immunological markers in this thesis provide some evidence of differences in immunoregulatory mechanisms directed against *N.battus* and trichostrongylid nematodes. The identified responder and non-responder lambs using *Nematodirus* FECs from the middle, end and overall season demonstrated no significant differences in IgE bearing cell numbers at any time point of the initial grazing season. This finding is in contrast to those of Chapter 5 and suggests that IgE bearing cells may not necessarily be a suitable supplementary marker of responsiveness against nematode infections when identifying responders and non-responders based on their *Nematodirus* egg counts.

Generally there appeared to be a direct relationship between the numbers of peripheral eosinophils and responsiveness against *N.battus*. Identified 'early' responder and non-responder lambs (using *Nematodirus* FECs from the middle of the season) had significantly higher peripheral eosinophil counts than non-responder lambs from late June to early August ($p<0.05$). Correlations between FEC and eosinophil numbers also showed a strong association between these two parameters in the identified end season responders. Additionally, analysis of the correlation plots showing mean FEC versus mean peripheral eosinophil count (Figure 6.6) confirmed that the majority of the identified *Nematodirus* end season non-responders had above mean FEC and below mean eosinophil numbers. Results suggest so far that it would be useful to select for responder and non-responder lambs based on mid season *Nematodirus* FEC levels and both mid and end season eosinophil numbers.

Analysis of the performance data suggested that there was only a weak association between responsiveness against *N.battus* and weight gain. Analysis of correlation plots displaying mean egg output and mean cumulative weight gain (Figure 6.7) showed that the majority of identified *Nematodirus* end season

responder lambs displayed below mean FEC levels and above mean cumulative weight gain. The majority of the end season non-responder lambs also had egg counts above the mean and below mean weight gain. Despite these trends in the responder and non responder lambs overall there was a low unfavourable correlation between *Nematodirus* FEC and production in the *Nematodirus* responder animals which would mitigate against using *N.battus* FECs as a phenotypic marker for selection.

No significant correlations were observed between cumulative weight gain and eosinophil levels or numbers of IgE bearing cells within the selected *Nematodirus* responder and non-responder lambs based on the whole season and mid season selection. A significant positive correlation was observed, however, between the cumulative weight gain and numbers of IgE bearing cells in the responder lambs selected on the basis of their *Nematodirus* FECs from the end of the season.

Data derived from the limited number of immunological parameters examined in this chapter cannot be used to provide evidence of commonality in the immune responses directed against *Nematodirus* and GI trichostrongyles it does provide some evidence of differences in how responsive and non responsive lambs react to the two types of nematode. Further research using identified responder and non-responder animals is needed in order to determine whether the regulatory mechanisms are truly discrete.

Chapter 7

Selection of Responder and Non-Responder Scottish Blackface Lambs using Immunological Parameters

7.1 INTRODUCTION

To date, the principal benefit sought when selecting animals for responsiveness against gastrointestinal nematode infections has been prophylaxis, with selected animals producing fewer eggs and hence over time reducing the challenge from pasture. Previous studies within this thesis using FEC as the phenotypic marker of responsiveness have demonstrated some potential parasitological benefits of this approach (chapters 4 and 5). Whatever parameters are used as selection criteria, should also be cheap, simple to use and most importantly should accurately identify animals that are not only resistant to gastrointestinal nematodes but are also productive. Using FECs as the sole selection criteria has a number of drawbacks including their relative costliness since they do not lend themselves to automation and an inability to discriminate within groups of animals with low egg counts. Immunological parameters have the potential to provide cheap markers of individual responsiveness since many of the assays can be automated.

Results from chapter five have suggested that IgE bearing cells and eosinophils may be important in the immune response to nematode infection whereas parasite specific antibodies have not shown to be as significant.

This current chapter therefore uses the IgE bearing cells and eosinophils independently, to select responder and non-responder lambs to determine whether they respond differently with respect to faecal egg output and weight gain.

7.2 MATERIALS AND METHODS

The methods used to determine FEC, weight gain, eosinophil and IgE bearing cell counts are described in chapter five.

Lambs were ranked in order of their cumulative resistance to nematode infection as determined by their IgE bearing cell or eosinophil counts for the end of the grazing season (7^{th} September – 17^{th} October, 2001). The ten lambs that had the highest overall IgE bearing cell/eosinophil count were termed "responders" and the animals with the lowest IgE bearing cell/eosinophil count were termed "non-responders". Data from chapter 5 (5.3) demonstrating the responders and non-responders selected using FEC is included in this result chapter for comparative reasons.

7.3 RESULTS

7.3.1 Selected responder and non-responder lambs using IgE bearing cells

Table 7.1 shows the eartag numbers of the identified responders and non-responders using their IgE bearing cell rankings from the end of the season.

End Season	
R	NR
690	631
697	652
744	719
830	746
835	783
900	894
960	909
963	945
1007	1033
1099	1115

Table 7.1 Animals selected as responders (R) and non-responders (NR) using their IgE bearing cell numbers from the end of the season.

7.3.2 IgE bearing cells of selected responder and non-responder lambs using IgE bearing cell rankings

The average IgE bearing cell counts of the selected responder and non-responder lambs from the end of the grazing season are shown in Figure 7.1. The average IgE bearing cell count of the whole flock was included on the graph.

The % IgE bearing cells of the responder animals increase from August onwards reaching approximately 30% in October. The % IgE bearing cells of the non-responder animals remain low (below 5%) throughout the grazing season.

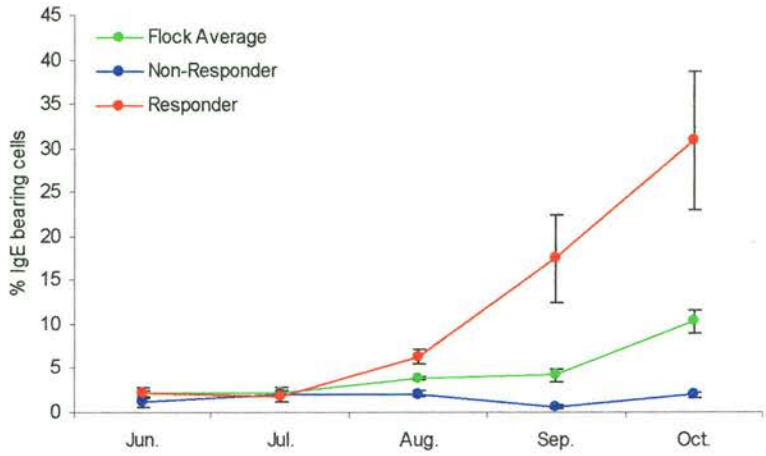


Figure 7.1 Average % IgE bearing cells (±SEM) for the selected responder and non-responder lambs using the IgE bearing cell rankings.

7.3.3 Trichostrongylid FECs of selected responder and non-responder lambs using IgE bearing cell rankings

Figure 7.2 shows the average FEC for the selected responder and non-responder lambs using the IgE bearing cell rankings from the end of the grazing season. The average FEC of the flock was included on each graph. The egg counts of both the responder and non-responder animals followed a similar pattern across the grazing season.

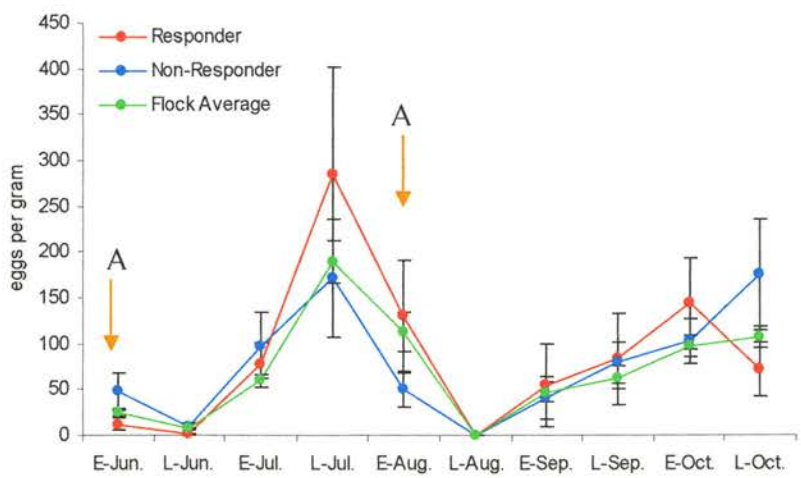


Figure 7.2
Average trichostrongylid FEC (±SEM) for the selected responder and non-responder lambs using the IgE bearing cell rankings (A = Anthelmintic treatment).

Egg counts of both groups of lambs peaked in late July with the responders displaying a slightly higher egg count than the non-responders (~300epg versus ~175epg, respectively). The egg counts of both groups of animals subsequently decreased even before anthelmintic treatment was administered, reaching zero levels in late August. The egg counts of both groups then gradually increased to ~100epg in October.

7.3.4 Cumulative weight gain of selected responder and non-responder lambs using IgE bearing cell rankings

The average cumulative weight gain of the selected responder and non-responder animals using the IgE bearing cell rankings from the end of the grazing season are shown in Figure 7.3. A steady increase in weight was observed in both groups of lambs from June to October. Responder lambs gained more weight than the non-responders over the whole of the grazing season, however significant differences were not demonstrated.

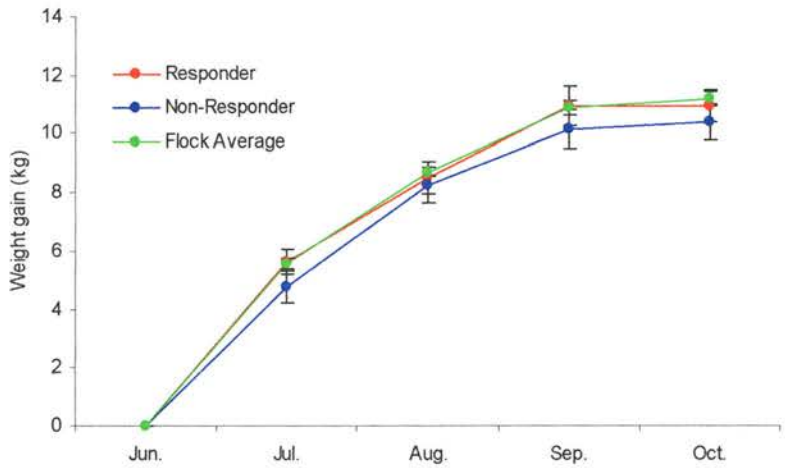


Figure 7.3 Average cumulative weight gains (±SEM) for the selected responder and non-responder lambs using the IgE bearing cell rankings.

7.3.5 Selected responder and non-responder lambs using eosinophil counts

Table 7.2 shows the eartag numbers of the identified responders and non-responders using eosinophil rankings from the end of the season.

End Season	
R	NR
697	699
771	746
782	781
827	807
830	808
852	813
856	866
960	877
1082	900
1099	977

Table 7.2 Animals selected as responders (R) and non-responders (NR) using their eosinophil counts from the end of the season.

7.3.6 Eosinophil counts of selected responder and non-responder lambs using eosinophil rankings

The average eosinophil counts of the selected responder and non-responder lambs from the end of the grazing season are shown in Figure 7.4. The average eosinophil count of the whole flock was included on the graph.

The eosinophil numbers of the responder animals began to increase from the beginning of the season through to early September reaching a count of approximately 40 x 10^9 /litre. Eosinophil numbers subsequently declined in late September and then increased again to counts of ~ 40x10^9/litre. The non-responder group of lambs displayed a very low eosinophil count throughout the grazing season.

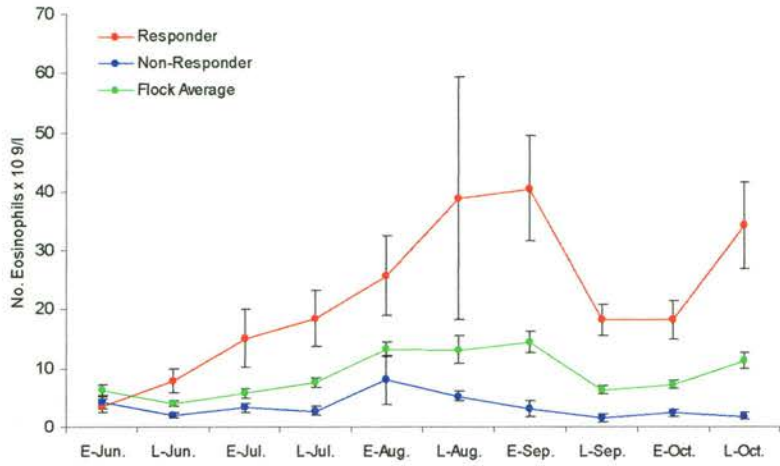


Figure 7.4 Average eosinophil count (±SEM) for the selected responder and non-responder lambs using the eosinophil rankings.

7.3.7 Trichostrongylid FECs of selected responder and non-responder lambs using eosinophil rankings

Figure 7.5 shows the average FEC for the selected responder and non-responder lambs using the eosinophil rankings from the end of the grazing season. The average FEC of the flock was included on each graph.

The egg counts of both the responder and non-responder animals followed a similar pattern across the grazing season with egg counts of both groups of lambs peaking in late July with the non-responders displaying a higher egg count than the responders (~200epg versus ~90epg, respectively). The egg counts of both groups of animals subsequently decreased following anthelmintic treatment reaching zero levels in late August. The egg counts of both groups then gradually increased to ~70epg. The responder lambs displayed lower FEC levels than the non-responders during the middle and end part of the grazing season.

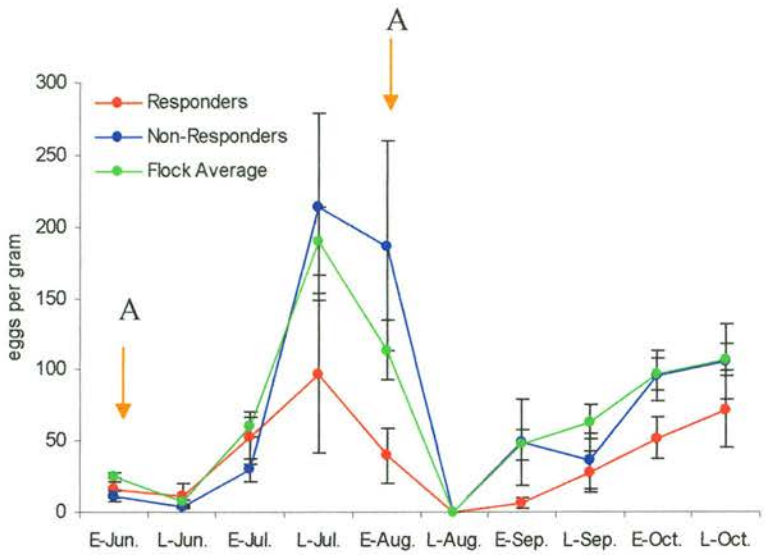


Figure 7.5 Average trichostrongylid FEC (±SEM) for the selected responder and non-responder lambs using the eosinophil rankings (A= Anthelmintic treatment).

7.3.8 Cumulative weight gain of selected responder and non-responder lambs using eosinophil rankings

The average cumulative weight gain of the selected responder and non-responder animals using the eosinophil rankings from the end of the grazing season are shown in Figure 7.6. A steady increase in weight was observed in both groups of lambs from June to October. Responder lambs gained more weight than the non-responders over most of the grazing season, however there were no significant differences in cumulative weight gain between the two groups.

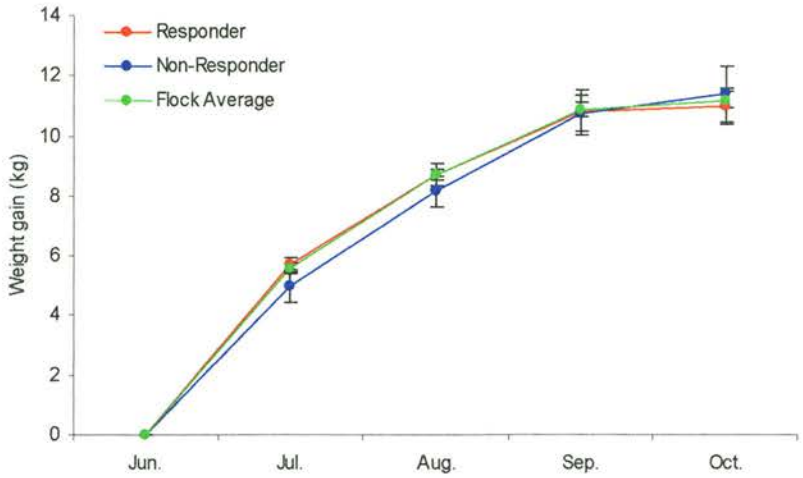


Figure 7.6 Average cumulative weight gains (±SEM) for the selected responder and non-responder lambs using the eosinophil rankings.

7.3.9 Selected responder and non-responder lambs using FECs

Table 7.3 shows the eartag numbers of the ten identified responders and non-responders using their FEC rankings from the end of the season. Moreover, the previously selected responder and non-responder lambs using IgE bearing cell counts and eosinophils are shown in this table for comparative study.

Four lambs appear on the IgE bearing cell and eosinophil responder list but only one non-responder is seen on both of these lists. The animals selected using the IgE bearing cell rankings and the FEC rankings shared two responder and two non-responder lambs in common. Three responders and one non-responder lamb selected using the eosinophil rankings were also selected using the FEC rankings.

End Season					
FEC		IgE Bearing cells		Eosinophils	
R	NR	R	NR	R	NR
740	631	690	631	697	699
744	779	697	652	771	746
850	783	744	719	782	781
852	861	830	746	827	807
857	877	835	783	830	808
1012	888	900	894	852	813
1043	898	960	909	856	866
1082	924	963	945	960	877
1099	978	1007	1033	1082	900
1104	1007	1099	1115	1099	977

Table 7.3 Animals selected as responders and non-responders using their FEC, IgE bearing cell counts and peripheral eosinophil counts from the end of the season.

7.3.10 Trichostrongylid FECs of responder and non-responder lambs identified using FEC rankings

The average FEC for the selected responder and non-responder lambs using the FEC rankings from the end of the grazing season are shown in Figure 7.7. The average FEC of the flock was included on each graph. The egg counts of both the responder and non-responder animals followed a similar pattern across the grazing season. Egg counts of both groups of lambs peaked in late July with the non-responders displaying a higher egg count than the responders (~450epg versus ~100epg, respectively). The egg counts of both groups of animals subsequently decreased even before anthelmintic treatment was administered, reaching zero levels in late August. The egg counts of the non-responders then increased to ~300epg in October whilst the responder animals remained at zero for the rest of the season.

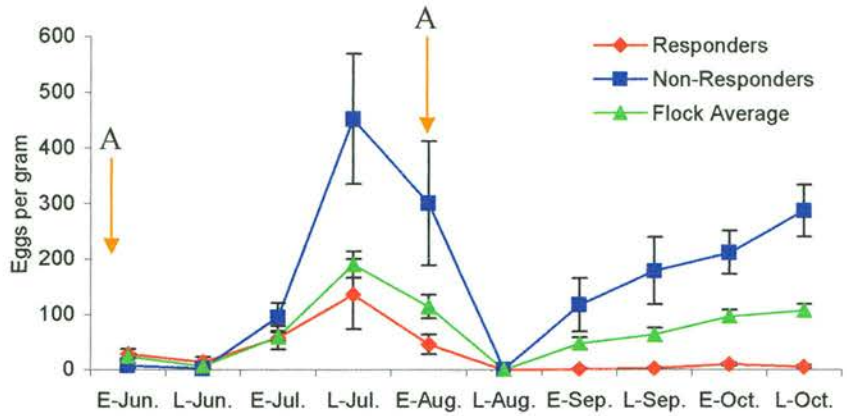


Figure 7.7 Average trichostrongylid FEC (±SEM) for the selected responder and non-responder lambs using the FEC rankings (A = Anthelmintic treatment).

7.3.11 IgE bearing cells of responder and non-responder lambs identified using FEC rankings

Figure 7.8 shows the average IgE bearing cell counts of the FEC selected responder and non-responder lambs from the end of the grazing season. The average IgE bearing cell count of the whole flock was included on the graph.

The % IgE bearing cells of the responder and non-responder animals began to increase gradually from August onwards with the responder lambs demonstrating higher IgE bearing cell numbers by the end of the season.

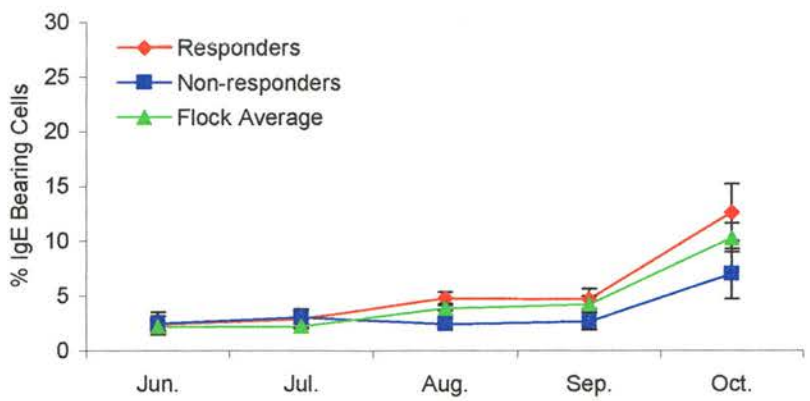


Figure 7.8 Average % IgE bearing cells (±SEM) for the selected responder and non-responder lambs using the FEC rankings.

7.3.12 Cumulative weight gain of responder and non-responder lambs identified using FEC rankings

Figure 7.9 shows the average cumulative weight gain of the selected responder and non-responder animals using the FEC rankings from the end of the grazing season. A steady increase in weight was observed in both groups of lambs from June to October. Responder lambs gained more weight than the non-responders over the whole of the grazing season. Responder lambs gained significantly more weight than the non-responders during the last part of the grazing season ($p<0.05$).

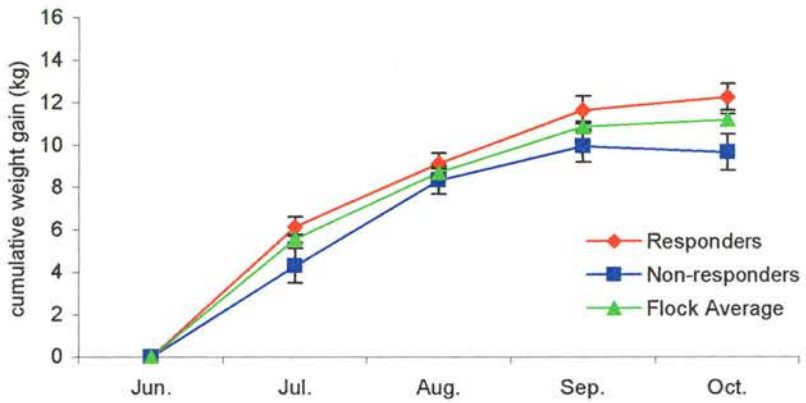


Figure 7.9 Average cumulative weight gains (±SEM) for the responder and non-responder lambs identified the FEC rankings.

7.3.13 Average across season egg count of responder animals selected using FEC, IgE bearing cells and eosinophils

The flock of lambs was estimated to have produced around 1719 million eggs across the grazing season. Table 7.4 shows the percentage of the total pasture contamination that the responders and non-responders selected using each parameter were estimated to have produced during the grazing season.

Selection Parameter	% Trichostrongylid Pasture contamination	
	Responders	Non-Responders
FECs	4	24
IgE bearing cells	11	12
Eosinophils	5	11

Table 7.4 Pasture contamination produced by responder and non-responder lambs over the grazing season

7.4 Discussion

The results from this chapter examining the relative benefits of using two immunological markers as phenotypic markers for responsiveness show that, unlike FEC selection, they confer less prophylactic benefit and have little or no positive impact on productivity. Only one responder lamb (1099) identified using FECs was identified using IgE bearing cells or eosinophils. Similar findings were also made with regard to non-responsive animals. Individual lambs identified as being responsive using trichostrongylid FECs were not the same individuals identified using IgE bearing cells or eosinophils.

Moreover, there may be inherent costs and difficulties associated with both markers. For example IgE bearing cells are non-specific markers to *T.circumcincta* infection and therefore other diseases might cause similar effects on the lamb's IgE bearing cell numbers. Additionally, the IgE bearing cell assay is very complex and costly and in its current format can only be used as a research tool. Eosinophil analysis is a cheaper and easier method than the IgE bearing cell assay, however, they have a similar drawback to the IgE bearing cells with regard to their non-specificity to *T.circumcinta* infections.

It is important to discover markers of responsiveness that have the ability to efficiently select responder lambs to nematode infection as there are penalties of mis-identifying responders. These penalties are relatively high when selecting for responsive rams because each ram will be expected to cover as many as 30 ewes and could produce up to 60 offspring at each mating season. These lambs will carry his genes so if the incorrect ram is selected then the rate of progress in selection programmes may be delayed. The effect would be less damaging for ewe selection since one ewe will only provide her genes to one or two lambs per breeding season.

In this study, the lamb flock produced an estimated 1719 million eggs during the grazing season (see Chapter 5). The responder lambs selected using the FECs from the end of the season accounted for only 4% of this total pasture contamination and whereas the responder lambs identified using the IgE bearing cells and eosinophils were responsible for 11% and 5% of the total pasture contamination, respectively. The non-responder group of lambs selected using FECs accounted for approximately 24% of pasture contamination demonstrating an approximate two-fold higher egg output across the season in comparison to the non-responders selected using both IgE

bearing cells and eosinophils. Since responsiveness is a heritable characteristic and appears to be relatively stable between seasons, identifying non-responsive animals at an early stage might allow targeted prophylactic anthelmintic treatments. Neither low IgE bearing cell numbers nor peripheral eosinophil counts appeared to provide the means of identifying the non-responder animals.

The data generated within this chapter suggest that it is perhaps more useful to consider the immunological markers, IgE bearing cells and eosinophils, as having the potential to increase the sensitivity of the selection process using FEC as the principal selection criterion. Additionally, since only a very limited number of immunological markers for responsiveness have been examined at this stage the possibility that there maybe other markers can not be ruled out.

Chapter 8

IgE and IgA activity against third stage and fourth stage *T.circumcincta* larvae in Texel lambs

8.1 INTRODUCTION

Previous studies have shown that different breeds of sheep may respond to nematode infections in different ways. For example, the study described in Chapter 4 has shown that IgE antibody responses appear to be of importance in the responsiveness of Greyface x Suffolk lambs against nematode infections. However, Chapter 5 has indicated that IgA antibody responses may be useful indicators of responsiveness against parasites in Scottish Blackface lambs. This study will evaluate the importance of both IgE and IgA antibody responses against nematodes in a third breed, namely Texel lambs born in 1998, 1999 and 2000. Additionally, the IgE and IgA activity against both third stage and fourth stage *T.circumcincta* larvae will be measured to determine if the IgE and IgA responses using both these stages of parasite show evidence of the same mechanisms.

8.2 MATERIALS AND METHODS

Serum samples used in this study were acquired from a previous project that was carried out by scientists at Roslin Institute, Glasgow University and Moredun Research Institute in 1998 – 2001.

8.2.1 Animals

Measurements were recorded on a flock of Texel lambs naturally infected from contaminated pastures at Roslin Institute's Blythbank farm. The pastures were predominantly infected with the strongyle *T.circumcincta*. The lambs were born between days 56 and 129 of each year, with the mean lambing day being 82.

8.2.2 Parasitological Parameters

Lambs were faecal sampled *per rectum* on three occasions each year, in July, August and September i.e when the lambs were 5, 6, and 7 months of age, on average, in 1998-2000. Faecal consistencies were monitored as described in 2.2.2. FECs were determined using a flotation method described in 2.2.3. Each sampling took place 4 weeks after anthelmintic treatment. Lambs grazed the same pastures during the duration of the measurement period. Lambs from all of the three years, were ranked in order of their cumulative resistance to nematode infection as

determined by their individual FECs for July to September. The ten lambs that had the lowest egg count as a whole were termed "responders" and the ten animals with the highest egg count were termed "non-responders".

8.2.3 Production Parameters

Blood samples were collected from the lambs on the final FEC sampling date for each year and serum was stored at –20°C for subsequent analysis (see 2.3).

8.2.4 Parasite specific ELISAs using S300 purified *T.circumcincta* L_3 and whole L_4 antigen

Parasite specific IgE and IgA ELISAs were carried out using the serum collected from the lambs over the grazing seasons (see 2.4.2). ELISA plates were coated with either prepared S300 purified *T.circumcincta* L_3 antigen at 1μg/ml or whole larval *T.circumcincta* L_4 antigen at the same concentration (see 2.2.7). For the parasite specific IgE ELISAs the secondary antibody used was anti-IgE 2F1 at 1/1000 dilution, and for IgA the secondary antibody used was anti-IgA at 1/2000 dilution, as described previously.

8.2.5 Statistical analysis

Statistical analysis was employed as described in 2.7.

8.3 RESULTS

8.3.1 Correlations between FEC and IgE and IgA antibody

For each year (98-00), it was determined whether there were any strong associations between FEC and the serum IgE and IgA antibody levels using both L_3 and L_4 *T.circumcincta* antigens. These correlations were examined for the whole flock and for the identified responders and non-responders. Table 8.1 displays the correlation results for the year 1998. No significant associations between FEC and IgE and IgA antibodies were observed using the L_3 or L_4 antigen within the whole flock or in the selected responder and non-responder lambs. However, within the whole flock, the FEC levels had a tendency to be low when IgE levels using the L_4 antigen were high although these associations were not significant.

1998	IgE L_3 v FEC		IgE L_4 v FEC		IgA L_3 v FEC		IgA L_4 v FEC	
	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value
Whole Flock	0.04	0.77	-0.22	0.08	-0.09	0.50	-0.03	0.84
Responder	0.47	0.17	0.25	0.49	0.27	0.46	-0.28	0.43
Non-Responder	0.15	0.69	0.16	0.67	0.28	0.43	0.02	0.96

Rho (ρ) = Spearman's correlation

Table 8.1 Correlations of the whole flock and selected responders and non-responders between FEC and IgE and IgA antibody titre using the L_3 and L_4 *T.circumcincta* antigen in 1998

Figure 8.1 demonstrates a plot showing FEC against IgE antibody of the whole flock and the selected responder and non-responder lambs for 1998 using the L_3 *T.circumcincta* antigen. The mean FEC and the mean IgE antibody levels of the whole flock were illustrated on the plot by a vertical and horizontal line, respectively. This was employed to determine if the lower FEC of responder lambs was associated with higher IgE levels than the flock as a whole and whether the higher FEC of non-responder lambs was associated with lower IgE levels. Figure 8.1 showed that 50 % of the responders displayed a higher IgE level and lower FEC and 80% of the non-responders demonstrated a higher FEC and lower IgE level than the mean values.

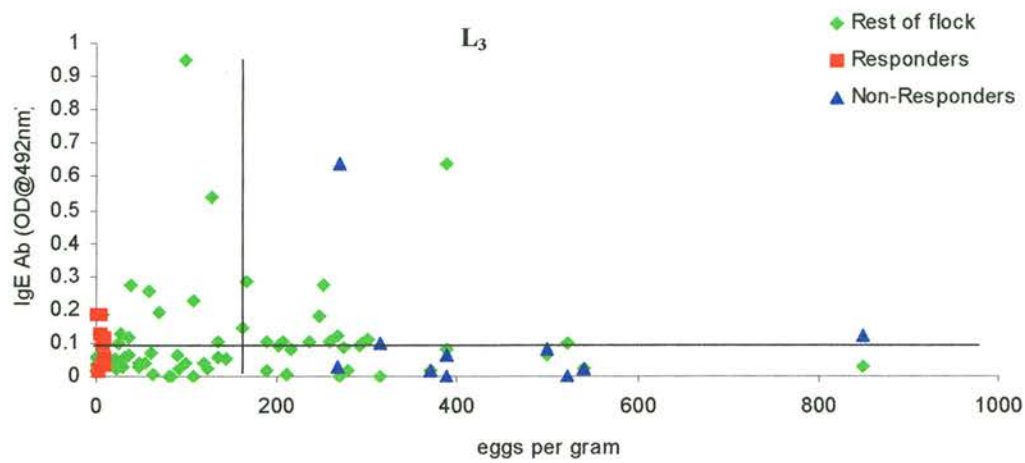


Figure 8.1 Plot showing FEC vs. IgE antibody of whole flock and selected responder and non-responder lambs from 1998 using the L_3 *T.circumcincta* antigen

Figure 8.2 demonstrates a plot showing FEC against IgE antibody of the whole flock and the selected responder and non-responder lambs for 1998 using the L_4 *T.circumcincta* antigen. This plot showed that 70 % of the responders displayed a higher IgE level and lower FEC and 90% of the non-responders demonstrated a higher FEC and lower IgE level than the mean values.

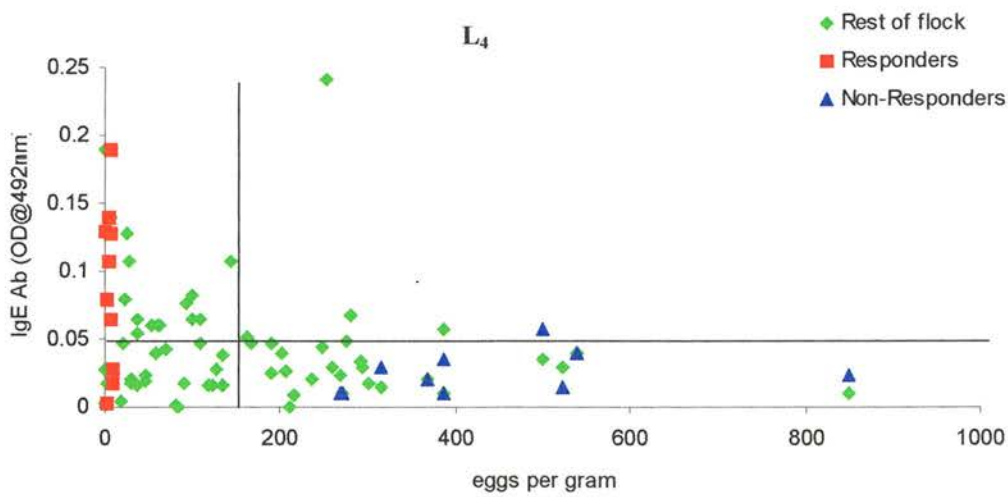


Figure 8.2 Plot showing FEC vs. IgE antibody of whole flock and selected responder and non-responder lambs from 1998 using the L_4 *T.circumcincta* antigen

Figure 8.3 demonstrates a plot showing FEC against IgA antibody of the whole flock and the selected responder and non-responder lambs for 1998 using the L_3 *T.circumcincta* antigen. This plot showed that 60 % of the responders displayed a higher IgA level and lower FEC and 60% of the non-responders demonstrated a higher FEC and lower IgA level than the mean values.

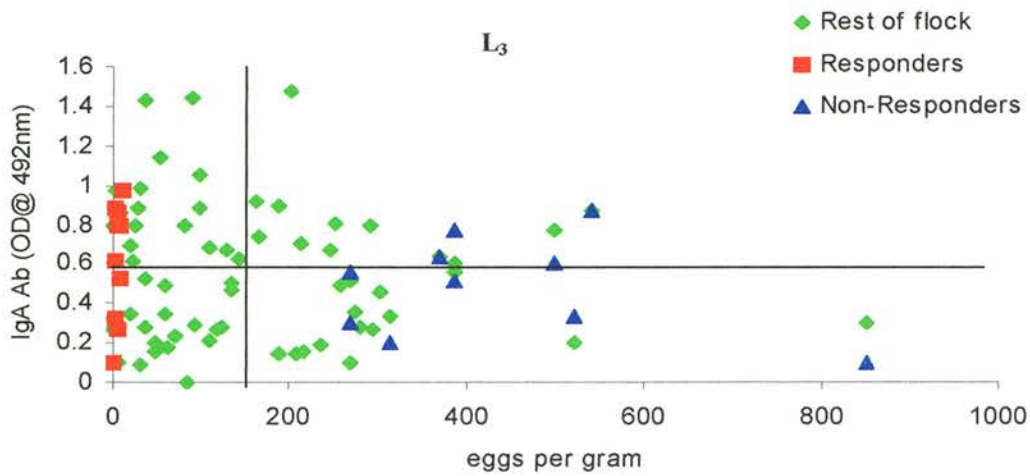


Figure 8.3 Plot showing FEC vs. IgA antibody of whole flock and selected responder and non-responder lambs from 1998 using the L_3 *T.circumcincta* antigen

Figure 8.4 demonstrates a plot showing FEC against IgA antibody of the whole flock and the selected responder and non-responder lambs for 1998 using the L_4 *T.circumcincta* antigen. This plot showed that 30 % of the responders displayed a higher IgA level and lower FEC and 70% of the non-responders demonstrated a higher FEC and lower IgA level than the mean values.

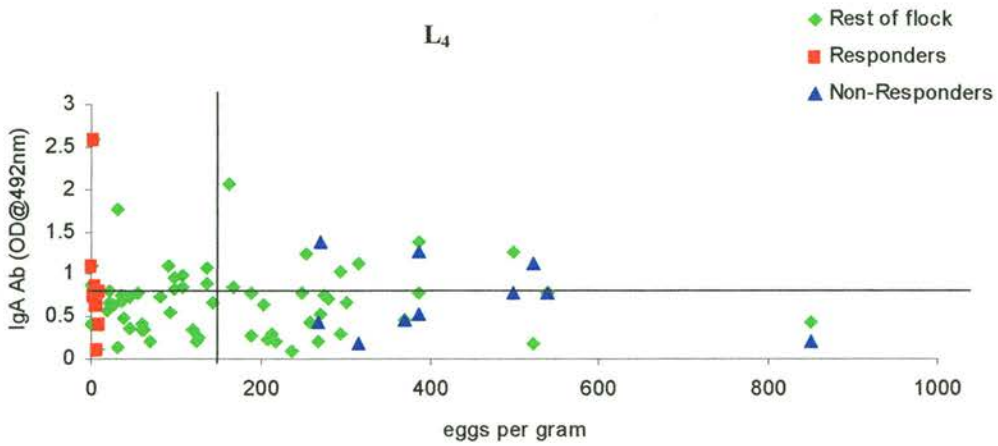


Figure 8.4 Plot showing FEC vs. IgA antibody of whole flock and selected responder and non-responder lambs from 1998 using the L_4 *T.circumcincta* antigen

In 1999 and 2000, similar correlation results to the year 1998 were observed between FEC and serum IgE and IgA antibodies. Table 8.2 and 8.3 display the correlations for these two consecutive years, respectively. No significant associations between FEC and IgE and IgA antibodies were observed using the L_3 or L_4 antigen in the selected responder and non-responder lambs. However, animals with low FEC tended to have a high IgE level using the L_4 antigen within the whole flock. This association was only significant in 1999 (ρ= -0.38, p=0.01).

1999	IgE L_3 v FEC		IgE L_4 v FEC		IgA L_3 v FEC		IgA L_4 v FEC	
	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value
Whole Flock	0.01	0.92	-0.38	0.01*	-0.15	0.30	-0.23	0.10
Responder	-0.15	0.69	-0.41	0.24	0.21	0.55	0.21	0.57
Non-Responder	-0.31	0.39	0.19	0.60	0.38	0.28	0.35	0.33

Table 8.2 Correlations of the whole flock and selected responders and non-responders between FEC and IgE and IgA antibody titre using the L_3 and L_4 *T.circumcincta* antigen in 1999

2000	IgE L_3 v FEC		IgE L_4 v FEC		IgA L_3 v FEC		IgA L_4 v FEC	
	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value	Rho (ρ)	p value
Whole Flock	-0.01	0.90	-0.11	0.28	0.03	0.75	0.01	0.95
Responder	-0.37	0.33	0.45	0.22	0.56	0.12	0.27	0.48
Non-Responder	-0.54	0.11	-0.48	0.16	-0.15	0.69	0.05	0.89

Table 8.3 Correlations of the whole flock and selected responders and non-responders between FEC and IgE and IgA antibody titre using the L_3 and L_4 *T.circumcincta* antigen in 2000.

Table 8.4 examines the within-class correlation between reactivity to L_3 and L_4 *T.circumcincta* antigens for the whole flock and selected responders and non-responders in 1998, 1999 and 2000. The results showed that IgA activity using L_3 antigen had a strong association with the IgA activity using L_4 antigen in the whole flock and in both the responder and non-responder lambs in all three years. These results were significant between the two variables with the whole flock and the non-responder lambs in all three years. Additionally, in 1999 and 2000, the responder lambs showed a significant correlation between the IgA antibody response using L_3 antigen and L_4 antigen. A significant association was also evident between the IgE activity using the L_3 antigen and the IgE activity using the L_4 antigen within the whole flock for each year but there was a lack of association between these two parameters with the responders and non-responders in 1998,1999 and 2000.

1998	IgE L_3 v IgE L_4		IgA L_3v IgA L_4	
	Rho	p	Rho	p
Whole Flock	0.28	0.03*	0.66	0.00*
Responder	0.59	0.07	0.39	0.26
Non-Responder	0.12	0.74	0.64	0.05*

1999	IgE L_3 v IgE L_4		IgA L_3v IgA L_4	
	Rho	p	Rho	p
Whole Flock	0.30	0.03*	0.70	0.00*
Responder	0.22	0.53	0.91	0.00*
Non-Responder	-0.14	0.70	0.78	0.01*

2000	IgE L_3 v IgE L_4		IgA L_3v IgA L_4	
	Rho	p	Rho	p
Whole Flock	0.31	0.00*	0.78	0.00*
Responder	-0.25	0.49	0.69	0.03*
Non-Responder	0.38	0.28	0.83	0.00*

Table 8.4 Correlation of the whole flock and selected responders and non-responders of IgE antibody titre using the L_3 and L_4 *T.circumcincta* antigen and IgA antibody titre using the L_3 and L_4 *T.circumcincta* antigen in 1998, 1999 and 2000

Figure 8.5 demonstrates plots of IgE activity using L_3 antigen against L_4 antigen and IgA activity using L_3 and L_4 antigen for the whole flock of lambs in 1998, 1999 and 2000. All three years show that a stronger relationship between IgA antibody responses using the L_3 antigen and L_4 antigen is seen compared to the IgE antibody responses using the L_3 and L_4 antigen.

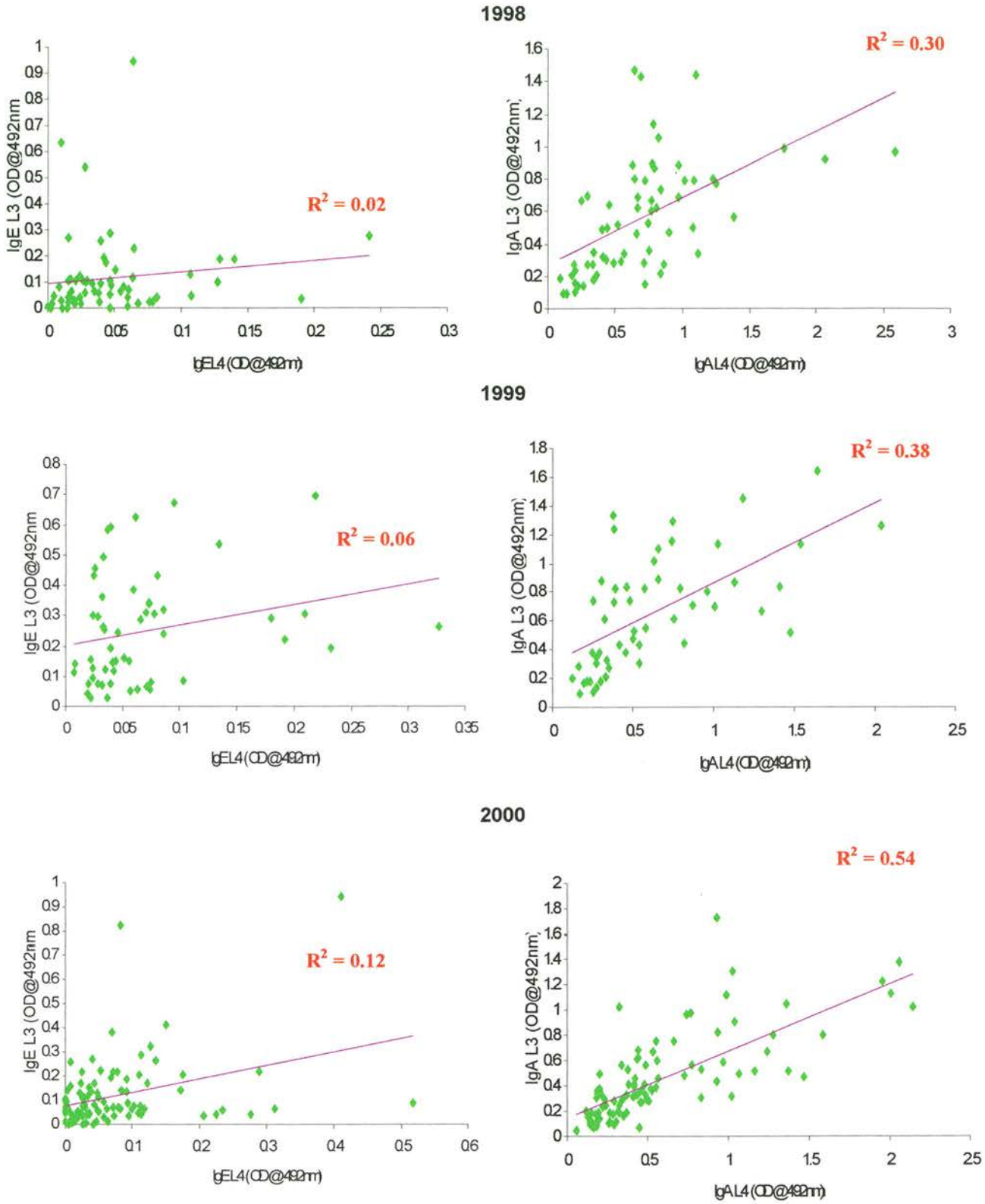


Figure 8.5 Plots of IgE activity using L_3 antigen against L_4 antigen and IgA activity using L_3 and L_4 antigen for the whole flock of lambs in 1998, 1999 and 2000

8.4 DISCUSSION

These results clearly show that both IgE and IgA antibody responses and egg counts in Texel lambs are almost unrelated in 1998,1999 and 2000. Within the lamb flock, however, there was a slight association, between IgE antibody response using the L_4 larval antigen and FEC during all three years. There was a tendency for the IgE levels to be high when the FECs were low, though this association was only significant in 1999. By plotting antibody activity against FEC and including the mean FEC and mean antibody levels, it was evident that in 1998 the majority of non-responders were located in the bottom right section of the quadrant, relating to a high FEC and low IgE antibody titre using the L_4 and L_3 antigens. The majority of the responders were situated in the top left section of the quadrant corresponding to a low egg output and high IgE antibody level using both L_4 and L_3 antigens. These results were very similar to the IgA antibody activity of the non-responders using both L_3 and L_4 antigen. The majority of the identified non-responder lambs were evident in the bottom right section of the quadrant (high FEC, low IgA antibody titre). However most of the responder lambs selected using either the L_3 or L_4 antigens were not located in the low FEC, high IgA antibody level sector. Although strong associations between antibody activity and FEC levels were not evident, it was apparent that the higher egg output of non-responder lambs for 1998 was associated with a lower antibody level than the mean of the flock. Therefore, the identified non-responders from these plots might be potential candidates for anthelmintic treatment.

Previous experiments indicated that parasite specific IgE and IgA antibody titres were not associated with a reduction in FEC in Scottish Blackface sheep during their first grazing season (Chapter 5). This may suggest that both the Texel and Scottish Blackface lambs behave similarly in their antibody responses against nematode infections.

This study additionally demonstrated that significant associations were present within the whole flock and in the responder and non-responder lambs between IgA antibody levels using the L_3 antigen and IgA antibody levels using the L_4 antigen in all three years. The responder and non-responder lambs also displayed significant

correlations between the IgE antibody activity using both of these antigens, however, the relationship was much weaker than the IgA reactivity to L_3 and L_4 antigens.

These results perhaps suggest that IgA antibody may be detecting the same antigens on both the *T.circumcincta* L_3 and L_4 larvae and that IgE antibodies were detecting different L_3 antigens that were not present on L_4. This could be because the surface antigens on third stage larvae are different to that on fourth stage larvae in relation to IgE antibody activity. Evidence of stage-specific antigens for parasites has been reported previously in the nematode cattle lungworm, *Dictyocaulus viviparus*. Antibody responses to the surface of adults, L_1 and eggs were specific for each stage of development (Britton *et al.*, 1993). Additionally, different life-stages of the abomasal cattle parasite *Ostertagia ostertagi* displayed different proteins as recognised by mucus antibodies (De Maere *et al.*, 2002). In Chapter 3 of this thesis, an IgE reactive immunodominant protein band was observed at 150kDa on the L_3 *T.circumcincta* antigen but a very faint IgE reactive immunodominant band was observed on the L_4 *T.circumcincta* antigen at 110kDa. Moreover, IgE and IgA antibodies both probably display different functions and mechanisms within the host-parasite system. It has been reported in several studies that IgE antibodies are probably associated with larval expulsion. Kooyman *et al.* (2002) suggests that in the case of *T.spiralis* infections, the highest intestinal IgE levels were found at a time of worm expulsion. The expulsion of gastrointestinal nematodes from the intestines of immune sheep has elements of an immediate hypersensitivity response, whereby nematode antigen binding to IgE-sensitised mast cells result in the release of chemical mediators which unfavourably affect nematode establishment. The specific effect of antigen with cell-bound IgE leads to activation of several non-specific effector mechanisms, one or all of which may result in nematode expulsion (Emery *et* al., 1997). Many of these IgE-related activities (Huntley, 1992; Miller, 1996) are known to occur in sheep and there is increasing support for the hypothesis that hypersensitivity reactions are responsible for expulsion of nematodes from immune animals and may contribute to the pathological changes associated with nematode resistance under field conditions (Douch *et* al., 1995). However, the exact role that hypersensitivity mechanisms play in the rejection of sheep nematodes has not been

fully elucidated and the processes that influence nematode survival, in the intestine remains unclear (Harrison *et al.* 1999).

In contrast, the IgA antibodies are possibly involved in other aspects of immunity such as inhibiting the growth, development and fecundity of the parasite (Stear *et al.*, 1999), or trapping them in mucus through altering the stability of the microenvironment (Harrison *et al.*, 1999). It has been reported that IgA antibody in mucus in immunised animals is significantly increased relative to levels in naïve sheep (Harrison *et al.*, 1999) and it has been suggested that these antibodies may reflect increased local production and/or plasma leakage, as a consequence of mediator release by mast cells causing increased vascular permeability (Steel *et al.*, 1990). Increases in gut antibody levels have been reported in previous studies and have been proposed as a possible mediator of immune damage to nematodes (Harrison *et al.*, 1999). It is not yet known whether mucus could cause complete elimination of the challenge dose of nematodes from the intestine of naïve recipient sheep.

Some of these identified effector mechanisms may not be directly attributable to the host. Changes in the microenvironment may be induced directly or indirectly by the parasites (Scott & McKellar, 1998) and the parasites might induce these changes directly or indirectly through immune responses. A physiologically mediated or immunophysiologically mediated effector mechanism could be used to explain 'turnover' (Michel, 1974) in *Teladorsagia* in which loss of adults is related to larval intake. The incoming larvae could change the microenvironment to the disadvantage of established adults and hasten loss. For example, infection with *Teladorsagia* spp. parasites is associated with nodular hyperplasia resulting in increased numbers of cells that produce both pepsinogen and mucus (mucopeptic cells) (Scott *et al.*, 1999).

Several hypotheses can be made to account for the cell changes of ostertagiosis. The first suggests that the cellular and consequently physiological changes in infection are the host responses induced by the physical presence of worms (Simpson *et al.*, 1997). Alternatively, the changes associated with helminth parasitism are caused by chemical substances released by the parasites themselves (Baker *et al.*, 1993).

In summary, this study has suggested that the antibody responses (IgE and IgA) to the L3 or L4 stages have little, or no, influence on the egg output of Texel lambs. This is in contrast to the earlier study with Blackface lambs, and may indicate important between breed differences in the mechanisms used to control worms.

Chapter 9

General Discussion

9.1 General Discussion

The studies within this thesis have examined important aspects of the host/parasite relationship with regard to the value of peripheral markers of immunoresponsivess against gastrointestinal nematodes at different stages of the grazing season. The relationship between level of larval challenge and disease is complex being influenced by a variety of host, parasite and environment dependant factors (Figure 9.1).

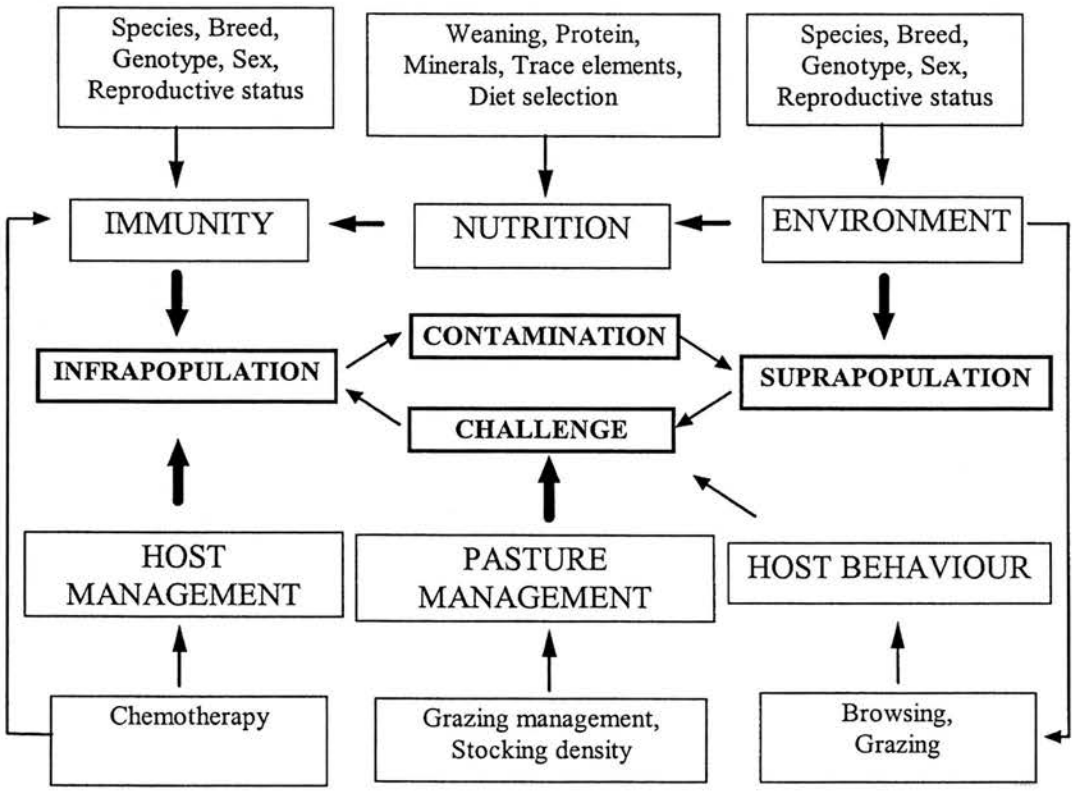


Figure 9.1 Interactive elements influencing parasite populations

Since acquired immunity is arguably the key factor regulating parasite populations, selecting lines of animals with an enhanced ability to regulate their infrapopulations has considerable potential as a means of combating disease. Breeding sheep which require minimal anthelmintic treatment to maintain high productivity and standards of welfare whilst exposed to challenge from pasture is an option which may help to: (a) satisfy consumer demands to limit drug usage in the

livestock industry (b) reduce costs of anthelmintic treatment to the farmers and (c) control anthelmintic resistance. However, the most appropriate breeding strategy to achieve this target is as yet unclear (Bisset & Morris, 1996). At present, FEC remains the principal factor, which can reliably be used to estimate host resistance. Knowledge of the heritability of traits that are important to the animal breeder is one of the essential parameters needed when planning a successful breeding program. Studies in South Africa have reported that the resistance of Merino sheep to natural infections with *H.contortus* as measured by FEC has a heritability of approximately 0.24 (Nieuwoudt *et al.*, 2002) whilst other studies using the same breed of sheep infected with *H.contortus* have demonstrated heritabilities that reach approximately 0.49. In Germany, Gauly *et al.* (2002) report heritabilities of approximately 0.35 in *H.contortus* infected Rhon sheep. Additionally, studies in New Zealand and Australia have demonstrated that the resistance of sheep to gastrointestinal parasites as evaluated by FEC has heritability in Romneys of approximately 0.35 following a natural challenge (Baker *et al.*, 1990) and moderate heritabilities (0.33-0.39) for this trait have been identified in Merino sheep (Windon *et* al., 1996; Woolaston *et al.*, 1990). FEC heritability studies in goats and cattle have also been employed and an estimated heritability of 0.33 was observed in Creole goats infected with *T.circumcuncta* and *T.colubriformis* (Mandonnet *et al.*, 2001) and Leighton *et al.*, (1989) found a heritability of FEC of approximately 0.29 in purebred Angus calves. Consequently, FEC is used currently in commercial breeding programmes both in Australia (*Nemesis*, CSIRO, 1994) and New Zealand (Worm FEC). In the UK, CBS Technologies and MLC's Signet Breeding Services are also offering a FEC analysis service enabling breeders to identify animals that are more resistant to nematode challenge.

FECs, however, may have their limitations as a direct measure of host resistance to nematode infection. Moreover, repeatability of FEC under field conditions has been reported to be highly variable (Bishop *et al.*, Animal Science, in press). This is of significance to the industry, as repeatability has a marked effect on the response to selection and the number of measurements needed for optimal genetic progress (Raadsma *et al*, 1998). Moreover, Claerebout *et al* (2000) have suggested that the parasitological parameters currently available for ruminants provide a relatively

crude measure of acquired immunity against gastrointestinal nematodes and to achieve a reasonable estimation of acquired immunity against gastrointestinal parasites, different parasitological and immunological parameters should be combined (Claerebout *et al.*, 2000).

A previous study (Douch *et al*, 1995) showed that using anti-worm antibody as the sole selection parameter produced heritabilities between 51-67% of those achieved using FEC as the sole selection criterion. However, this reduction in efficiency on the selection of antibody responses would, to a certain extent, be compensated for by the practical benefits that an ELISA has over measuring FEC. Additionally, it is possible that antibody responses may more directly reflect the sheep's ability to withstand nematode establishment (Douch *et al.*, 1995).

This current investigation has also provided evidence that there may be a potential to integrate other immunological parameters of responsiveness into selective breeding programmes in addition to FECs.

The principal aim of this research was to evaluate the importance of both IgE and IgA circulating antibodies in their application as markers of responsiveness against parasitic infection in sheep. In these studies the heritabilities of both these antibody responses for nematode resistance were not measured, the main aim being to gain an insight into the role of these antibodies in the immune response to nematode infection. One of the key findings from this research was the importance of breed. Peripheral parasite specific IgE antibodies appeared to be significant in the immune regulation of *T.circumcincta* infection in Greyface x Suffolk lambs (Chapter 4) but IgA seemed more important in Scottish Blackface lambs (Chapter 5). Other studies have also shown the importance of IgE (Huntley *et al.*, 1998a; Shaw *et al.*, 1998) and IgA (Stear *et al.*, 1995a; Strain *et al.*, 2002) in Greyface x Suffolk and Scottish Blackface lambs, respectively.

Although there have been a number of studies, the precise role played by IgE in immunoregulation has not been defined. IgE has been strongly involved with the immune rejection of incoming L_3 larvae in some parasite-host models (Bell *et al.*, 1992; Harrison *et al.*, 1999), via mast cell activity and a resultant hypersensitivity response (Huntley, 1992; Miller, 1996). Even though a role for parasite specific IgE antibodies in the responsiveness of Greyface x Suffolk lambs against *T.circumcincta*

infection was observed in this pilot study, the same IgE antibody response pattern was not evident in the Scottish Blackface lamb experiment (Chapter 5) where parasite specific IgA antibodies appeared to be more significant. Other studies using Scottish Blackface lambs infected with *Teladorsagia* have also demonstrated an anti-parasite function for parasite specific IgA antibody, defined by the relationship of low FEC, worm length and IgA antibody to adult antigens (Stear *et al.*, 1995b). These authors suggested that the main mechanism for the immune control of *T.circumcincta* in lambs in their first grazing season was facilitated through IgA antibodies, which affected the development and fecundity of established worms. Once again the exact role of IgA antibodies has not been precisely defined.

The production of these two different antibody responses (IgE and IgA) may imply that the immune control of *Teladorsagia* is a complex process involving two or more mechanisms, for example;

(a) an IgA mediated response involved with effector mechanisms influencing development and fecundity.

(b) an IgE mediated hypersensitivity response principally affecting incoming larvae.

Breed specific differences in peripheral antibody responses between hill (Scottish Blackface) and lowland breeds (Greyface x Suffolk) may have been naturally selected in response to differences in challenge faced by these types of sheep. In hill animals where stocking densities are low, regulating growth and fecundity of the parasites may be sufficient to protect the host population. In intensively reared lowland breeds exposed to high levels of challenge an ability to regulate incoming larvae may be more important. In summary, it can be suggested that antibody responses may be useful as an additional selection criterion in these breeds but further research is required into antibody heritabilities and the characterisation of the mechanisms associated with the parasite specific IgE and IgA response.

As mentioned previously, additional indicator traits are required to supplement the data provided by FECs for selecting sheep that are resistant to *T.circumcincta*. One such trait may include the IgE bearing cells; a novel marker investigated in this current study with the Scottish Blackface lambs. These IgE bearing cells were found predominantly among B-lymphocytes, which may be recognised as antigen

presenting cells important in presenting antigen to activated T-cells in peripheral tissues (Kapsenberg *et al.*, 1998; Robert & Kupper, 1999). In particular, B-cells bearing IgE bound to CD23 are very effective at presenting allergen to T-cells (Mudde *et al.*, 1996). IgE bearing cells measured in Chapter 5 represented the total IgE response rather than the parasite specific IgE response. This may account for the differences observed between total and parasite specific IgE antibodies in the Scottish Blackface lamb study. During a primary response to nematode infection, total IgE measured is associated with antigen presentation and parasite specific IgE is involved in the degranulation of other cells and producing antiparasite activity so does not rise until after challenge (Shaw *et al.*, 1998). These findings of Shaw *et al.* (1998) support the view that total IgE measured by the presence of IgE bearing cells, seemed to be more important as a determinant of responsiveness against nematodes than the peripheral parasite specific IgE antibodies in Scottish Blackface lambs.

Another immunological trait that could assist in establishing the resistance of a sheep to parasitic infection could be peripheral eosinophils. It has been reported that peripheral eosinophilia is mostly but not always elevated in sheep that are resistant to the nematodes *T.colubriformis* (Kimambo *et al.*, 1988; Dawkins *et al.*, 1989; Rothwell *et al.*, 1993) and *H.contortus* (Gill, 1991; Woolaston *et al.*, 1996) as well as sheep identified for resistance to natural infection in New Zealand (Douch *et al.*, 1996). Stear *et al.* (2002) have also shown that estimated heritabilities for eosinophil numbers in four and five month old Scottish Blackface lambs were 0.48 and 0.43 respectively and therefore, under defined conditions, eosinophil numbers may be a useful indicator of resistance to predominantly *T.circumcincta* infection. This present study also investigated the value of peripheral eosinophilia as an indicator of responsiveness to *T.circumcincta* in Scottish Blackface lambs. Eosinophil counts were markedly higher from July onwards in the identified responder lambs and their eosinophil numbers were negatively correlated with FECs from July onwards in the first grazing season. These results suggested that associations between eosinophil numbers and FECs were present in lambs that were approximately three months old. Douch *et al.* (1996) have suggested that any association between eosinophil numbers and FECs may only become apparent when eosinophil numbers are elevated. The results from this current study as well as from an investigation reported by Stear *et*

al. (2002) are in accordance with this suggestion. However, it is possible that there are circumstances when peripheral eosinophil counts may not consistently represent what is happening at the gastrointestinal mucosa. A low number of eosinophils in the circulation could result from factors such as reduced ability to produce eosinophils from stem cells, or enhanced ability to attract and retain them at the mucosa (Douch *et al.*, 1995). High eosinophil counts might also reflect other parasite pathogen contact within or on the host, for example, *Psoroptes ovis* (van den Broek *et al.*, 2003) and *Oestrus ovis* (Tabouret *et al.*, 2003).

Following anthelmintic treatment, eosinophil counts have a tendency to decrease quite suddenly and therefore if an association between FEC and eosinophils is only present when eosinophils are elevated then some care would be required if this immunological factor is to be employed as an indicator trait for the responsiveness to *T.circumcincta* (Stear *et al.*, 1995a). Eosinophils may be most beneficial in older lambs that have been continually exposed to infection and in the UK, it is these animals that are more inclined to be subject to selection (Stear *et al.*, 1995a).

Eosinophils can play a key role as effector cells in resistance to parasitic infection (Walsh, 1999; Meeusen & Balic, 2000; Behm & Ovington, 2000) or they may be a by-product of the processes required to control nematode infections (Stear *et al.*, 2002). The association present in this study between egg counts and eosinophil numbers could be because of either direct or indirect effects. It has been suggested that these results support the idea that an important mechanism of resistance to *T.circumcincta* infection in older lambs is the regulation of worm development, growth and fecundity (Stear *et al.*, 1999). In summary, responder lambs following a natural infection of gastrointestinal nematodes, predominantly *T.circumcincta*, tended to have higher peripheral eosinophil numbers and a reduced egg count from July onwards in the first grazing season. Stear *et al.* (2002) has reported a similar occurrence and speculated that the reduction in egg counts may be due to a decline in parasite fecundity as opposed to the presence of fewer *T.circumcincta*.
Although it has been shown that various immunological factors may be useful in selective breeding programmes of sheep it is vital to understand the impact of selection on the economically important traits of sheep, for example, liveweight gain, milk production, fertility and wool growth. Several studies have described selection

programmes based on naturally acquired field infections with predominantly *Teladorsagia* and *Trichostrongylus* spp and have demonstrated a positive association between resistance and production parameters (Windon & Dineen, 1984; Albers *et al.*, 1987; Woolaston, 1990). In contrast, studies in New Zealand have shown that although there are potentially significant benefits to be gained from breeding sheep for resistance to nematode infection, these benefits do not seem to be associated with large advantages in animal performance (Bisset *et al.*, 1997) and low unfavourable genetic correlations between faecal egg counts and production traits have been observed. Selecting solely for resistance may under certain circumstances, impose some production penalty, since many of the pathogenic effects associated with worm infection may have an immunopathogenic aetiology. One of the most important pathogenic effects seen in nematode infections is the effect on appetite. As reviewed by Coop *et al* (2001), *Trichostrongylus* infections can reduce appetite in growing lambs by 15-20%, which significantly impairs performance. Effects upon appetite may be attributable to either some direct 'worm' effect or may be indirect as a consequence of immune responses directed against the parasites. Recent evidence from New Zealand (Sykes, personal communication) suggests that the latter effect is more important. Ongoing studies in New Zealand have shown that immunosuppressant treatments with dexamethasone enables large *T.colubriformis* populations to survive but that these large populations do not affect appetite. These findings together with the New Zealand findings on the negative impact of selecting for host resistance (Bisset *et al.*, 1997) suggest that it may be more important to select for animals that are resilient, with an ability to perform well under challenge. However, as figure 9.1 shows, the relationship between challenge and contamination is also affected by a number of environmental factors. The extent of challenge faced by animals in a subtropical country such as New Zealand where animals can graze throughout the year might well be higher than that faced in countries like the UK where grazing only provides sufficient nutrients during the spring, summer and autumn months. For these reasons it seems reasonable to suggest that different regions and agroclimatic zones may require different approaches. In situations such as the UK where the extent and duration of challenge diminishes during the winter

months, the penalties associated with hyper-responsiveness may be of less importance than in countries where the level of challenge is high throughout the year.

In this study (Chapter 5), IgE bearing cells appear to be associated with a higher cumulative weight gain although significant associations were not observed to fully substantiate this. A lack of association was, however, observed between eosinophil numbers and the weight gain of lambs. Future studies will need to be employed to concentrate on the use of these two traits as indicators of responsiveness and their impact on sheep productivity. Additionally, it will be important to assess the significance of these immunological parameters in other breeds of sheep naturally infected with *T.circumcincta*.

Even though parasitological and immunological parameters have the potential to act as markers of resistance to nematode infection in sheep, it is clearly important to identify genetic markers for nematode resistance that can be used for selection purposes. It is now clear that many different genes regulate the immune response to infection with gastrointestinal nematodes, and evidence has been found for host genetic variation – some animals are more resistant or more tolerant to nematode infections than others (Behnke *et al.*, 2003). Inter-specific, inter-breed and intra-breed genetic differences are likely to exist. Comparative studies using sheep and goats have provided an example of inter-specific differences in responsiveness against nematodes with the former species being more susceptible (Huntley *et al.*, 1995). Inter-breed variation is important in the tropics where indigenous breeds of sheep that are locally adapted are likely to be superior to imported exotic genotypes. The exotic breeds however, may be beneficial in other ways and cross breeding can be utilised to enhance tolerance or resistance (Yadav *et al.*, 1993). Intra-breed differences have been widely utilised (Douch *et al.*, 1996; Windon, 1996, 1990; Woolaston *et al.*, 1996) using parasitological criteria for selection programmes. In the future, immunological and genetic markers will also be used as a means of selecting on farm and within breeds.

Between breed differences in peripheral markers of immunoresponsiveness in this study suggest that different genetic markers may be required for each breed. A similar situation exists for scrapie resistance where at least five different alleles have been shown to be important with substantial variation in breed distribution of PrP

alleles (Dawson *et al.*, 1998). The availability of genotyping methods for nematode resistance or susceptibility would offer some considerable advantages for the industry. Additional genetic markers that are currently being investigated include microsatellite markers, which can be used to identify those quantitative trait loci (QTL) responsible for resistance to gastrointestinal nematodes (Behnke *et al.*, 2003).

Any control measure intended to regulate parasite populations has the potential to lead to genetic change in the parasite population since it favours those individuals that are able to evade the control strategy. Chemical control strategies impose a high selection pressure and have in many cases led to the selection of anthelmintic resistant populations. Genetic improvement of host resistance may not exert such a high selection pressure since they are less effective and may allow 'susceptible' parasites to survive. If this is indeed the case then genetic strategies are likely to be more sustainable than many other control strategies for nematode infections. This may not be true, however for bacteria and viruses, where evolutionary change in the parasite population may eventually occur (Hammond, 2003).

It has been reported that selecting responder animals that are more capable of inducing an immune response to nematode infection than non-responders is very important in the future of selective breeding programmes. However, it is also of significance to be able to identify susceptible animals from a flock of sheep as demonstrated in the genetic selection studies of sheep for a high risk of scrapie (Dawson *et al.*, 1998). Selecting for susceptibility as a means of targeting only the most susceptible animals would have the advantage of reducing anthelmintic usage and thus reducing the pressure for drug resistance (Bisset & Morris, 1996).

The use of immunological traits in this study to evaluate the immunity of sheep against *T.circumcincta* has been based on their correlation with parasitological parameters such as FEC. Until the mechanisms of immunity are fully understood, parasitological parameters will probably be the most efficient way to estimate immunity against gastrointestinal nematodes. The existence of animals possessing extremes of responsiveness provides a powerful research tool for investigating the mechanisms of resistance to nematodes. Understanding the basis of the selected parameter in breeding programmes is essential for identifying predictive markers with resistance. Moreover, identifying the mechanisms involved in susceptibility to

the nematodes will allow the opportunity for immunological manipulations to induce resistance (Windon, 1990).

References

Abbott, E. M., Parkins, J. J., and Holmes, P. H. (1985). Influence of dietary protein on the pathophysiology of ovine haemonchosis in Finn Dorset and Scottish Blackface lambs given a single moderate infection. *Res.Vet.Sci.* **38**, 54-60.

Ahmad, A., Wang, C. H., and Bell, R. G. (1991). A role for IgE in intestinal immunity. Expression of rapid expulsion of *Trichinella spiralis* in rats transfused with IgE and thoracic duct lymphocytes. *J.Immunol.* **146**, 3563-3570.

Albers, G. A. and Gray, G. D. (1987). Breeding for worm resistance: a perspective. *Int.J.Parasitol.* **17**, 559-566.

Altaif, K. I. and Dargie, J. D. (1978). Genetic resistance to helminths. The influence of breed and haemoglobin type on the response of sheep to primary infections with *Haemonchus contortus*. *Parasitology* **77**, 161-175.

Ashman, K., Mather, J., Wiltshire, C., Jacobs, H. J., and Meeusen, E. (1995). Isolation of a larval surface glycoprotein from *Haemonchus contortus* and its possible role in evading host immunity. *Mol.Biochem.Parasitol.* **70**, 175-179.

Baker, D. G., Gershwin, L. J., and Hyde, D. M. (1993). Cellular and chemical mediators of type 1 hypersensitivity in calves infected with *Ostertagia ostertagi*: mast cells and eosinophils. *Int.J.Parasitol.* **23**, 327-332.

Baker, R.L., T.G. Watson, S.A. Bisset, A. Vlossoff, (1990). Breeding Romney sheep which are resistant to gastrointestinal parasites. *Proc. Austr. Assoc. Anim. Breed. Genet.* **50**: 417.

Bancroft, A. J. and Grencis, R. K. (1998). Th1 and Th2 cells and immunity to intestinal helminths. *Chem.Immunol.* **71**, 192-208.

Barger, I. A. (1987). Population regulation in trichostrongylids of ruminants. *Int.J.Parasitol.* **17** , 531-540.

Barger, I. A. (1989). Genetic resistance of hosts and its influence on epidemiology. *Vet.Parasitol.* **32**, 21-35.

Barger, I. A. (1993). Control of gastrointestinal nematodes in Australia in the 21st century. *Vet.Parasitol.* **46**, 23-32.

Barger, I. A. (1996). Prospects for integration of novel parasite control options into grazing systems. *Int.J.Parasitol.* **26**, 1001-1007.

Barger, I. A. (1999). The role of epidemiological knowledge and grazing management for helminth control in small ruminants. *Int.J.Parasitol.* **29**, 41-47.

Barrett, M (1997) PhD thesis, Moredun Research Institute & Glasgow University.

Bawden, R. J. (1969). A rapid technique for the recovery of strongyloid larvae from pasture samples. *Aust.Vet.J.* **45**, 228-230.

Beh, K. J. and Maddox, J. F. (1996). Prospects for development of genetic markers for resistance to gastrointestinal parasite infection in sheep. *Int.J.Parasitol.* **26**, 879-897.

Behm, C. A. and Ovington, K. S. (2000). The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. *Parasitol.Today* **16**, 202-209.

Behnke, J. M., Iraqi, F., Menge, D., Baker, R. L., Gibson, J., and Wakelin, D. (2003). Chasing the genes that control resistance to gastrointestinal nematodes. *J.Helminthol.* **77**, 99-110.

Beuzen, N. D., Stear, M. J., and Chang, K. C. (2000). Molecular markers and their use in animal breeding. *Vet.J.* **160**, 42-52.

Bisset, S. A. and Morris, C. A. (1996). Feasibility and implications of breeding sheep for resilience to nematode challenge. *Int J.Parasitol* **36**, 857-868.

Bisset, S. A., Vlassoff, A., West, C. J., and Morrison, L. (1997). Epidemiology of nematodosis in Romney lambs selectively bred for resistance or susceptibility to nematode infection. *Vet.Parasitol.* **70**, 255-269.

Blaxter, A., Mayer, S., Nicol, C., Wotton, P., Bailey, M., Dukes, P., Newton-Clarke, M., Stevens, K., Wilson, A. D., and Miller, B. (1989). MoD grant to Bristol veterinary school. *Vet.Rec.* **124**, 22.

Boluda, L. and Berrens, L. (1995). Do IgE-IgG complexes occur in the circulation? *Clin.Exp.Immunol.* **100**, 145-150.

Bowles, V. M., Brandon, M. R., and Meeusen, E. (1995) Characterization of local antibody responses to the gastrointestinal parasite Haemonchus contortus. *Immunology*, **13 (12)**, 1131-7.

Buddle, B. M., Jowett, G., Green, R. S., Douch, P. G., and Risdon, P. L. (1992). Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *Int.J.Parasitol.* **22**, 955-960.

Campbell, W. C. (1993). Ivermectin, an antiparasitic agent. *Med.Res.Rev.* **13**, 61-79.

Capron, M., Capron, A., Dessaint, JP., Torpier, G., Johansson, SG., Prin, L. (1981). Fc receptors for IgE on human and rat eosinophils. *J Immunol.* **126 (6)**, 2087-92.

Carlisle, M. S., McGregor, D. D., and Appleton, J. A. (1990). The role of mucus in antibody-mediated rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* **70**, 126-132.

Claerebout, E., Agneessens, Shaw, D. J., and Vercruysse, J. (1999). Larval migration inhibition activity in abomasal mucus and serum from calves infected with *Ostertagia ostertagi*. *Res.Vet.Sci.* **66**, 253-257.

Claerebout, E. and Vercruysse, J. (2000). The immune response and the evaluation of acquired immunity against gastrointestinal nematodes in cattle: a review. *Parasitology* **120 Suppl**, S25-S42.

Clunies Ross, I. (1932). Observations on the resistance of sheep to infestation by the stomach worm *Haemonchus contortus*. *Journal of the council for scientific and industrial research*, **5**, 73-80.

Colditz, I. G., Watson, D. L., Gray, G. D., and Eady, S. J. (1996). Some relationships between age, immune responsiveness and resistance to parasites in ruminants. *Int.J.Parasitol.* **26**, 869-877.

Connan, R. M. (1968). Studies on the worm populations in the alimentary tract of breeding ewes. *J.Helminthol.* **42**, 9-28.

Connan, R. M. (1972). The effect of host lactation on a second infection of Nippostrongylus brasiliensis in rats. *Parasitology* **64**, 229-233.

Coop, R. L., Huntley, J. F., and Smith, W. D. (1995). Effect of dietary protein supplementation on the development of immunity to *Ostertagia circumcincta* in growing lambs. *Res.Vet.Sci.* **59**, 24-29.

Coop, R. L. and Kyriazakis, I. (1999). Nutrition-parasite interaction. *Vet.Parasitol.* **84**, 187-204.

Coop, R. L. and Kyriazakis, I. (2001). Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol.* **17**, 325-330.

Crowle, P. K. (1983). Mucosal mast cell reconstitution and *Nippostrongylus brasiliensis* rejection by W/Wv mice. *J.Parasitol.* **69**, 66-69.

Cutts, L. and Wilson, R. A. (1997). The protein antigens secreted in vivo by adult male Schistosoma mansoni. *Parasitology* **114 (Pt 3)**, 245-255.

Darwish, R. A., Sanad, M. M., and Youssef, S. M. (1996). Immunization against *Trichinella spiralis* using antigens from different life-cycle stages experimental study in mice. *J.Egypt.Soc.Parasitol.* **26**, 19-26.

Dawkins, H. J., Windon, R. G., and Eagleson, G. K. (1989). Eosinophil responses in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis*. *Int.J.Parasitol.* **19**, 199-205.

Dawson, M., Hoinville, L. J., Hosie, B. D., and Hunter, N. (1998). Guidance on the use of PrP genotyping as an aid to the control of clinical scrapie. Scrapie Information Group. *Vet.Rec.* **142**, 623-625.

Dell, A., Haslam, S. M., Morris, H. R., and Khoo, K. H. (1999). Immunogenic glycoconjugates implicated in parasitic nematode diseases. *Biochim.Biophys.Acta* **1455**, 353-362.

Dhar, D. N. and Sharma, R. L. (1981). Immunization with irradiated larvae against *Dictyocaulus filaria* in young lambs. *Vet.Parasitol.* **9**, 125-131.

Dineen, J. K., Gregg, P., and Lascelles, A. K. (1978). The response of lambs to vaccination at weaning with irradiated *Trichostrongylus colubriformis* larvae: segregation into 'responders' and 'non-responders'. *Int.J.Parasitol.* **8**, 59-63.

Dobson, C. and Bawden, R. J. (1974) Studies on the immunity of sheep to *Oesophagostomum columbianum*: effects of low-protein diet on resistance to infection and cellular reactions in the gut. *Parasitology*, **69 (2)**, 239-55.

Donald, A. D., Morley, F. H., Waller, P. J., Axelsen, A., Dobson, R. J., and Donnelly, J. R. (1982). Effects of reproduction, genotype and anthelmintic treatment of ewes on *Ostertagia* spp. populations. *Int.J.Parasitol.* **12**, 403-411.

Douch, P. G., Harrison, G. B., Buchanan, L. L., and Greer, K. S. (1983). In vitro bioassay of sheep gastrointestinal mucus for nematode paralysing activity mediated by substances with some properties characteristic of SRS-A. *Int.J.Parasitol.* **13**, 207-212.

Douch, P. G., Harrison, G. B., Elliott, D. C., Buchanan, L. L., and Greer, K. S. (1986). Relationship of gastrointestinal histology and mucus antiparasite activity with the development of resistance to trichostrongyle infections in sheep. *Vet.Parasitol.* **20**, 315-331.

Douch, P. G. C., Green, R. S., Morris, C. A., and Hickey, S. M. (1995). Genetic factors affecting antibody responses to four species of nematode parasite in Romney ewe lambs. *Int.J.Parasitol.* **25**, 823-828.

Douch, P. G. C., Green, R. S., Morris, C. A., McEwans, J. C., and Windon, R. G. (1996). Phenotypic Markers for Selection of Nematode-resistant Sheep. *Int.J.Parasitol.* **26**, 899-911.

Dunsmore, J. D. (1966). Influence of host reproduction on numbers of trichostrongylid nematodes in the European rabbit, Oryctolagus cuniculus (L.). *J.Parasitol.* **52**, 1129-1133.

East, I. J., Fitzgerald, C. J., and Berrie, D. A. (1993) Oesophagostomum radiatum: the effect of different adjuvants on vaccination with a partially purified protective antigen. *Int.J.Parasitol.* **23 (2)**, 221-9.

Else, K. J., Hultner, L., and Grencis, R. K. (1992). Cellular immune responses to the murine nematode parasite *Trichuris muris*. II. Differential induction of TH-cell subsets in resistant versus susceptible mice. *Immunology* **75**, 232-237.

Emery, D. L., Wagland, B. M., and McClure, S. J. (1993). Rejection of heterologous nematodes by sheep immunized with larval or adult *Trichostrongylus colubriformis*. *Int.J.Parasitol.* **23**, 841-846.

Flint, M. L. (1998). Pests of the Garden and Small Farm: A Grower's Guide to Using Less Pesticide, 2nd ed. Oakland: Univ. Calif. Div. *Agric. Nat. Res. Publ.* 3332.

Gasbarre, L. C., Leighton, E. A., and Sonstegard, T. (2001) Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. *Vet.Parasitol.*, **98(1-3)**, 51-64

Gauly, M., Kraus, M., Vervelde, L., van Leeuwen, M. A., and Erhardt, G. (2002). Estimating genetic differences in natural resistance in Rhon and Merinoland sheep following experimental *Haemonchus contortus* infection. *Vet.Parasitol.* **106**, 55-67.

Geerts, S., Coles, G.C., Gryseels, B. (1997). A resistance in human helminths: learning from the problems with worm control in livestock. *Parasitol. Today.* **13 (4)** 149-151.

Gibbs, H. C. (1986). Hypobiosis in parasitic nematodes--an update. *Adv.Parasitol.* **25**, 129-174.

Gibson, T. E. and Parfitt, J. W. (1972). The effect of age on the development by sheep of resistance to *Trichostrongylus colubriformis*. *Res.Vet.Sci.* **13**, 529-535.

Gill, H. S. (1991). Genetic control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunol.* **13**, 617-628.

Gill, H. S., Watson, D. L., and Brandon, M. R. (1992). In vivo inhibition by a monoclonal antibody to CD4+ T cells of humoral and cellular immunity in sheep. *Immunology* **77**, 38-42.

Gill, H. S., Gray, G. D., Watson, D. L., and Husband, A. J. (1993a). Isotype-specific antibody responses to *Haemonchus contortus* in genetically resistant sheep. *Parasite Immunol.* **15**, 61-67.

Gill, H. S., Colditz, I. G., and Watson, D. L. (1993b). Immune responsiveness of lambs selected for resistance to haemonchosis. *Res.Vet.Sci.* **54**, 361-365.

Gill, H. S., Husband, A. J., Watson, D. L., and Gray, G. D. (1994). Antibody-containing cells in the abomasal mucosa of sheep with genetic resistance to Haemonchus contortus. *Res.Vet.Sci.* **56**, 41-47.

Gilleard, J. S., Duncan, J. L., and Tait, A. (1995). An immunodominant antigen on the *Dictyocaulus viviparus* L3 sheath surface coat and a related molecule in other strongylid nematodes. *Parasitology* **111 (Pt 2)**, 193-200.

Gray, G. D. and Gill, H. S. (1993). Host genes, parasites and parasitic infections. *Int.J.Parasitol.* **23**, 485-494.

Grencis, R. K. (1996). T cell and cytokine basis of host variability in response to intestinal nematode infections. *Parasitology* **112 Suppl**, S31-S37.

Grencis, R. K. (1997). Th2-mediated host protective immunity to intestinal nematode infections. *Philos.Trans.R.Soc.Lond B Biol.Sci.* **352**, 1377-1384.

Gronvold, J., Henriksen, S. A., Larsen, M., Nansen, P., and Wolstrup, J. (1996). Biological Control: Aspects of biological control - with special reference to arthropods, protozoans and helminths of domesticated animals. *Vet. Parasitol.* **64**, 47-64.

Hagan, P. (1993). IgE and protective immunity to helminth infections. *Parasite Immunol.* **15**, 1-4.

Hall, J. G., Hopkins, J., and Orlans, E. (1977). Migration pathways of lymphoid cells with reference to the gut, immunoglobulins and lymphomata [proceedings]. *Biochem.Soc.Trans.* **5**, 1581-1583.

Hammond K. (2003). Genetics and animal health. *Food and agriculture organization of the united nations.*

Harrison, G. B., Pulford, H. D., Gatehouse, T. K., Shaw, R. J., Pfeffer, A., and Shoemaker, C. B. (1999). Studies on the role of mucus and mucosal hypersensitivity reactions during rejection of *Trichostrongylus colubriformis* from the intestine of immune sheep using an experimental challenge model. *Int.J.Parasitol.* **29**, 459-468.

Hertzberg, H., Larsen, M., and Maurer, V. (2002). [Biological control of helminths in grazing animals using nematophagous fungi]. *Berl Munch.Tierarztl.Wochenschr.* **115**, 278-285.

Holmes, P. H. (1985). Pathogenesis of trichostrongylosis. *Vet.Parasitol.* **18**, 89-101.

Houdijk, J. G., Kyriazakis, I., Coop, R. L., and Jackson, F. (2001). The expression of immunity to *Teladorsagia circumcincta* in ewes and its relationship to protein nutrition depend on body protein reserves. *Parasitology* **122**, 661-672.

Huntley, J. F., Gibson, S., Knox, D., and Miller, H. R. (1986). The isolation and purification of a proteinase with chymotrypsin-like properties from ovine mucosal mast cells. *Int.J.Biochem.* **18**, 673-682.

Huntley, J. F., Gibson, S., Brown, D., Smith, W. D., Jackson, F., and Miller, H. R. (1987). Systemic release of a mast cell proteinase following nematode infections in sheep. *Parasite Immunol.* **9**, 603-614.

Huntley, J. F. (1992). Mast cells and basophils: a review of their heterogeneity and function. *J.Comp Pathol.* **107**, 349-372.

Huntley, J. F., Patterson, M., MacKellar, A., Jackson, F., Stevenson, L. M., and Coop, R. L. (1995). A comparison of the mast cell and eosinophil responses of sheep and goats to gastrointestinal nematode infections. *Res.Vet.Sci.* **58**, 5-10.

Huntley, J. F., Schallig, H. D., Kooyman, F. N., MacKellar, A., Jackson, F., and Smith, W. D. (1998a). IgE antibody during infection with the ovine abomasal nematode, *Teladorsagia circumcincta*: primary and secondary responses in serum and gastric lymph of sheep. *Parasite Immunol.* **20**, 565-571.

Huntley, J. F., Schallig, H. D., Kooyman, F. N., MacKellar, A., Millership, J., and Smith, W. D. (1998b). IgE responses in the serum and gastric lymph of sheep infected with *Teladorsagia circumcincta*. *Parasite Immunol.* **20**, 163-168.

Huntley, J. F., Redmond, J., Welfare, W., Brennan, G., Jackson, F., Kooyman, F., and Vervelde, L. (2001). Studies on the immunoglobulin E responses to *Teladorsagia circumcincta* in sheep: purification of a major high molecular weight allergen. *Parasite Immunol.* **23**, 227-235.

Jackson, F., Miller, H. R., Newlands, G. F., Wright, S. E., and Hay, L. A. (1988). Immune exclusion of *Haemonchus contortus* larvae in sheep: dose dependency, steroid sensitivity and persistence of the response. *Res.Vet.Sci.* **44**, 320-323.

Jackson, F. (1993). Anthelmintic resistance--the state of play. *Br.Vet.J.* **149**, 123-138.

Jacobs, H. J., Wiltshire, C., Ashman, K., and Meeusen, E. N. (1999). Vaccination against the gastrointestinal nematode, *Haemonchus contortus*, using a purified larval surface antigen. *Vaccine* **17**, 362-368.

Jarrett, E. E. and Urquhart, G. M. (1969). Immunological unresponsiveness to helminth parasites. 3. Challenge of rats previously infected at an early age with *Nippostrongylus brasiliensis*. *Exp.Parasitol.* **25**, 245-257.

Jarrett, E. E. and Miller, H. R. (1982). Production and activities of IgE in helminth infection. *Prog.Allergy* **31**, 178-233.

Jasmer, D. P. and McGuire, T. C. (1991). Protective immunity to a blood-feeding nematode (*Haemonchus contortus*) induced by parasite gut antigens. *Infect.Immun.* **59**, 4412-4417.

Jasmer, D. P., Perryman, L. E., and McGuire, T. C. (1996). *Haemonchus contortus* GA1 antigens: related, phospholipase C-sensitive, apical gut membrane proteins encoded as a polyprotein and released from the nematode during infection. *Proc.Natl.Acad.Sci.U.S.A* **93**, 8642-8647.

Jeffcoate, I. A., Wedrychowicz, H., Fishwick, G., Dunlop, E. M., Duncan, J. L., and Holmes, P. H. (1992). Pathophysiology of the periparturient egg rise in sheep: a possible role for IgA. *Res.Vet.Sci.* **53**, 212-218.

Jenkins, R. E., Taylor, M. J., Gilvary, N. J., and Bianco, A. E. (1998). Tropomyosin implicated in host protective responses to microfilariae in onchocerciasis. *Proc.Natl.Acad.Sci.U.S.A* **95**, 7550-7555.

Jessop, N. S. (1997). Protein metabolism during lactation. *Proc.Nutr.Soc.* **56**, 169-175.

Jones, W. O., Window, R. G., Steel, J. W., and Outteridge, P. M. (1990). Histamine and leukotriene concentrations in duodenal tissue and mucus of lambs selected for high and low responsiveness to vaccination and challenge with *Trichostrongylus colubriformis*. *Int.J.Parasitol.* **20**, 1075-1079.

Jones, W. O., Emery, D. L., McClure, S. J., and Wagland, B. M. (1994). Changes in inflammatory mediators and larval inhibitory activity in intestinal contents and mucus during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int.J.Parasitol.* **24**, 519-525.

Jordon, R. M. and Marten, G. C. (1968). Effect of weaning, age at weaning and grain feeding on the performance and production of grazing lambs. *J.Anim Sci.* **27**, 174-177.

Jungery, M. and Ogilvie, B. M. (1982). Antibody response to stage-specific *Trichinella spiralis* surface antigens in strong and weak responder mouse strains. *J.Immunol.* **129 A**, 839-843.

Kapsenberg, M. L., Hilkens, C. M., Wierenga, E. A., and Kalinski, P. (1998). The role of antigen-presenting cells in the regulation of allergen- specific T cell responses. *Curr.Opin.Immunol.* **10**, 607-613.

Kawakami, T. and Galli, S. J. (2002). Regulation of mast-cell and basophil function and survival by IgE. *Nat.Rev.Immunol.* **2**, 773-786.

Kezar, W. W. and Church, D. C. (1979). Ruminal changes during the onset and recovery of induced lactic acidosis in sheep. *J.Anim Sci.* **49**, 1161-1167.

Kimambo, A. E., MacRae, J. C., and Dewey, P. J. (1988). The effect of daily challenge with *Trichostrongylus colubriformis* larvae on the nutrition and performance of immunologically-resistant sheep. *Vet.Parasitol.* **28**, 205-212.

Knox, D. P., Redmond, D. L., and Jones, D. G. (1993). Characterization of proteinases in extracts of adult *Haemonchus contortus*, the ovine abomasal nematode. *Parasitology* **106 (Pt 4)**, 395-404.

Knox, D. P. and Smith, W. D. (2001). Vaccination against gastrointestinal nematode parasites of ruminants using gut-expressed antigens. *Vet.Parasitol.* **100**, 21-32.

Knox, M. and Steel, J. (1996). Nutritional enhancement of parasite control in small ruminant production systems in developing countries of South-East Asia and the Pacific. *Int.J.Parasitol.* **26**, 963-970.

Kooyman, F. N., Van Kooten, P. J., Huntley, J. F., MacKellar, A., Cornelissen, A. W., and Schallig, H. D. (1997). Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to Haemonchus contortus infection. *Parasitology* **114 (Pt 4)**, 395-406.

Kuranu, F.N., McGuire, T.C., Davis, W.C., Besser, T.E. and Jasmer, D.P., 1997. $CD4^+$ T lymphocytes contribute to protective immunity induced in sheep and goats by *Haemonchus contortus* gut antigens. *Parasitol. Immunol.* **19**, pp. 435–445

Lacey, E. (1988). The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int.J.Parasitol.* **18**, 885-936.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (259): 680-5.

Larsen, M., Nansen, P., Gronvold, J., Wolstrup, J., and Henriksen, S. A. (1997). Biological control of gastro-intestinal nematodes--facts, future, or fiction? *Vet.Parasitol.* **72**, 479-485.

Larsen, M. (1999). Biological control of helminths. *Int.J.Parasitol.* **29**, 139-146.

Lawrence, C. E., Paterson, J. C., Higgins, L. M., MacDonald, T. T., Kennedy, M. W., and Garside, P. (1998). IL-4-regulated enteropathy in an intestinal nematode infection. *Eur.J.Immunol.* **28**, 2672-2684.

Le Jambre, L. F., Martin, P. J., and Jarrett, R. G. (1982). Comparison of changes in resistance of Haemonchus contortus eggs following withdrawal of thiabendazole selection. *Res.Vet.Sci.* **32**, 39-43.

Leighton, E. A., Murrell, K. D., and Gasbarre, L. C. (1989). Evidence for genetic control of nematode egg-shedding rates in calves. *J.Parasitol.* **75**, 498-504.

Longbottom, D., Redmond, D. L., Russell, M., Liddell, S., Smith, W. D., and Knox, D. P. (1997). Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein complex from adult *Haemonchus contortus*. *Mol.Biochem.Parasitol.* **88**, 63-72.

Love, R. J., Ogilvie, B. M., and McLaren, D. J. (1976). The immune mechanism which expels the intestinal stage of *Trichinella spiralis* from rats. *Immunology* **30**, 7-15.

Luffau, G., Pery, P., and Charley, J. (1981). [Immune response in sheep experimentally infected with *Haemonchus contortus*, Comparative study in male and female (author's transl)]. *Ann.Rech.Vet.* **12**, 173-181.

Lumley, A. M. and Lee, D. L. (1981). *Nippostrongylus brasiliensis* and *Nematodirus battus*: changes in numbers and weight during the course of infection. *Exp.Parasitol.* **52**, 183-190.

Maizels, R. M. and Page, A. P. (1990). Surface associated glycoproteins from *Toxocara canis* larval parasites. *Acta Trop.* **47**, 355-364.

Maizels, R. M. and Holland, M. J. (1998). Parasite immunology: pathways for expelling intestinal helminths. *Curr.Biol.* **8**, R711-R714.

Mandonnet, N., Aumont, G., Fleury, J., Arquet, R., Varo, H., Gruner, L., Bouix, J., and Khang, J. V. (2001). Assessment of genetic variability of resistance to gastrointestinal nematode parasites in Creole goats in the humid tropics. *J.Anim Sci.* **79**, 1706-1712.

Marriner, S. (1986) Anthelmintic drugs. *Vet.Record*, **118(7)**, 181-4.

McClure, S. J., Emery, D. L., Wagland, B. M., and Jones, W. O. (1992). A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *Int.J.Parasitol.* **22**, 227-234.

McKeand, J. B., Duncan, J. L., Urquhart, G. M., and Kennedy, M. W. (1996). Isotype-specific antibody responses to the surface-exposed antigens of adult and larval stages of *Dictyocaulus viviparus* in infected and vaccinated calves. *Vet.Parasitol.* **61**, 287-295.

Meeusen, E. N. and Balic, A. (2000). Do eosinophils have a role in the killing of helminth parasites? *Parasitol.Today* **16**, 95-101.

Michel, J. F. (1974). Arrested development of nematodes and some related phenomena. *Adv.Parasitol.* **12**, 279-366.

Michel, J. F., Lancaster, M. B., and Hong, C. (1976). The resumed development of arrested *Ostertagia ostertagi* in experimentally infected calves. *J.Comp Pathol.* **86**, 615-619.

Michel, J. F., Lancaster, M. B., and Hong, C. (1979). The effect of age, acquired resistance, pregnancy and lactation on some reactions of cattle to infection with *Ostertagia ostertagi*. *Parasitology* **79**, 157-168.

Michel, J. F. (1985). Strategies for the use of anthelmintics in livestock and their implications for the development of drug resistance. *Parasitology* **90 (Pt 4)**, 621-628.

Miller, H. R. and Huntley, J. F. (1982). Protection against nematodes by intestinal mucus. *Adv.Exp.Med.Biol.* **144**, 243-245.

Miller, H. R., Jackson, F., Newlands, G., and Appleyard, W. T. (1983). Immune exclusion, a mechanism of protection against the ovine nematode *Haemonchus contortus*. *Res.Vet.Sci.* **35**, 357-363.

Miller, H. R. (1984). The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Vet.Immunol.Immunopathol.* **6**, 167-259.

Miller, H. R. (1987). Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. *Parasitology* **94 Suppl**, S77-100.

Miller, H. R. (1996). Mucosal mast cells and the allergic response against nematode parasites. *Vet.Immunol.Immunopathol.* **54**, 331-336.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J.Immunol.* **136**, 2348-2357.

Mosmann, T. R. and Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu.Rev.Immunol.* **7**, 145-173.

Mudde, G. C., Reischul, I. G., Corvaia, N., Hren, A., and Poellabauer, E. M. (1996). Antigen presentation in allergic sensitization. *Immunol.Cell Biol.* **74**, 167-173.

Munn, E. A., Greenwood, C. A., and Coadwell, W. J. (1987). Vaccination of young lambs by means of a protein fraction extracted from adult *Haemonchus contortus*. *Parasitology* **94 (Pt 2)**, 385-397.

Munn, E. A., Smith, T. S., Graham, M., Greenwood, C. A., Tavernor, A. S., and Coetzee, G. (1993). Vaccination of merino lambs against haemonchosis with membrane-associated proteins from the adult parasite. *Parasitology* **106 (Pt 1)**, 63-66.

Muto, R., Imai, S., Tezuka, H., Furuhashi, Y., and Fujita, K. (2001). The biological activity of ABA-1-like protein from *Ascaris lumbricoides*. *J.Med.Dent.Sci.* **48**, 95-104.

Nawa, Y., Ishikawa, N., Tsuchiya, K., Horii, Y., Abe, T., Khan, A. I., Bing, S., Itoh, H., Ide, H., and Uchiyama, F. (1994). Selective effector mechanisms for the expulsion of intestinal helminths. *Parasite Immunol.* **16**, 333-338.

Neilson, J. T. (1975) Failure to vaccinate lambs against *Haemonchus contortus* with functional metabolic antigens identified by immunoelectrophoresis. *Int.J.Parasitol.*, **5(4)**,427-30.

Newlands, G. F., Miller, H. R., and Jackson, F. (1990). Immune exclusion of *Haemonchus contortus* larvae in the sheep: effects on gastric mucin of immunization, larval challenge and treatment with dexamethasone. *J.Comp Pathol.* **102**, 433-442.

Newton, S. E. (1995). Progress on vaccination against Haemonchus contortus. *Int.J.Parasitol.* **25**, 1281-1289.

Ngwenya, B. Z. (1977). Response of nonsensitized and sensitized lactating mice to infection with *trichinella spiralis*. *Int.J.Parasitol.* **7**, 41-45.

Nieuwoudt, S. W., Theron, H. E., and Kruger, L. P. (2002). Genetic parameters for resistance to *Haemonchus contortus* in Merino sheep in South Africa. *J.S.Afr.Vet.Assoc.* **73**, 4-7.

O'Malley, K. E., Sloan, T., Joyce, P., and Baird, A. W. (1993). Type I hypersensitivity reactions in intestinal mucosae from rats infected with *Fasciola hepatica*. *Parasite Immunol.* **15**, 449-453.

O'Sullivan, B. M. and Donald, A. D. (1970). A field study of nematode parasite populations in the lactating ewe. *Parasitology* **61**, 301-315.

O'Sullivan, B. M. and Donald, A. D. (1973). Responses to infection with *Haemonchus contortus* and *Trichostrongylus colubriformis* in ewes of different reproductive status. *Int.J.Parasitol.* **3**, 521-530.

Ogilvie, B. M. and Jones, V. E. (1973). Immunity in the parasitic relationship between helminths and hosts. *Prog.Allergy* **17**, 93-144.

Paterson, S., Wilson, K., and Pemberton, J. M. (1998). Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population. *Proc.Natl.Acad.Sci.U.S.A* **95**, 3714-3719.

Patterson, D. M., Jackson, F., Huntley, J. F., Stevenson, L. M., Jones, D. G., Jackson, E., and Russel, A. J. (1996). The response of breeding doses to nematodiasis: segregation into "responders" and "non-responders". *Int.J.Parasitol.* **26**, 1295-1303.

Paxton, W. A., Yazdanbakhsh, M., Kurniawan, A., Partono, F., Maizels, R. M., and Selkirk, M. E. (1993). Primary structure of and immunoglobulin E response to the repeat subunit of gp15/400 from human lymphatic filarial parasites. *Infect.Immun.* **61**, 2827-2833.

Pritchard, D. I. (1993). Immunity to helminths: is too much IgE parasite--rather than host-protective? *Parasite Immunol.* **15**, 5-9.

Pritchard, D. I., Brown, A., and Toutant, J. P. (1994). The molecular forms of acetylcholinesterase from *Necator americanus* (Nematoda), a hookworm parasite of the human intestine. *Eur.J.Biochem.* **219**, 317-323.

Raadsma, H. W., Gray, G. D., and Woolaston, R. R. (1998). Breeding for disease resistance in Merino sheep in Australia. *Rev.Sci.Tech.* **17**, 315-328.

Rahman, W. A. and Collins, G. H. (1992). An association of faecal egg counts and prolactin concentrations in sera of periparturient Angora goats. *Vet.Parasitol.* **43**, 85-91.

Rainbird, M. A., Macmillan, D., and Meeusen, E. N. (1998). Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin- 5. *Parasite Immunol.* **20**, 93-103.

Redmond, D. L., Knox, D. P., Newlands, G., and Smith, W. D. (1997). Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*. *Mol.Biochem.Parasitol.* 77-87.

Robert, C. and Kupper, T. S. (1999). Inflammatory skin diseases, T cells, and immune surveillance. *N.Engl.J.Med.* **341**, 1817-1828.

Rothwell, T. L. and Griffiths, D. A. (1977). Comparison of the kinetics of expulsion of *Trichostrongylus colubriformis* from previously uninfected, reinfected, and vaccinated guinea pigs. *J.Parasitol.* **63**, 761-762.

Rothwell, T. L. (1989). Immune expulsion of parasitic nematodes from the alimentary tract. *Int.J.Parasitol.* **19**, 139-168.

Rothwell, T. L., Windon, R. G., Horsburgh, B. A., and Anderson, B. H. (1993). Relationship between eosinophilia and responsiveness to infection with *Trichostrongylus colubriformis* in sheep. *Int.J.Parasitol.* **23**, 203-211.

Santiago, M. L., Hafalla, J. C., Kurtis, J. D., Aligui, G. L., Wiest, P. M., Olveda, R. M., Olds, G. R., Dunne, D. W., and Ramirez, B. L. (1998). Identification of the *Schistosoma*

japonicum 22.6-kDa antigen as a major target of the human IgE response: similarity of IgE-binding epitopes to allergen peptides. *Int.Arch.Allergy Immunol.* **117** , 94-104.

Sato, M. N., Carvalho, A. F., Silva, A. O., MacIel, M., Jr., Fusaro, A. E., and Duarte, A. J. (1998). Oral tolerance induced to house dust mite extract in naive and sensitized mice: evaluation of immunoglobulin G anti-immunoglobulin E autoantibodies and IgG-IgE complexes. *Immunology* **95**, 193-199.

Schallig, H. D., van Leeuwen, M. A., and Hendrikx, W. M. (1995). Isotype-specific serum antibody responses of sheep to *Haemonchus contortus* antigens. *Vet.Parasitol.* **56**, 149-162.

Schallig, H. D., van Leeuwen, M. A., and Cornelissen, A. W. (1997). Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. *Parasite Immunol.* **19**, 447-453.

Schallig, H. D. (2000). Immunological responses of sheep to *Haemonchus contortus*. *Parasitology* **120 Suppl**, S63-S72.

Schuurs, A. H. and Verheul, H. A. (1990). Effects of gender and sex steroids on the immune response. *J.Steroid Biochem.* **35**, 157-172.

Schwaiger, F. W., Gostomski, D., Stear, M. J., Duncan, J. L., McKellar, Q. A., Epplen, J. T., and Buitkamp, J. (1995). An ovine major histocompatibility complex DRB1 allele is associated with low faecal egg counts following natural, predominantly *Ostertagia circumcincta* infection. *Int.J.Parasitol.* **25**, 815-822.

Scott, I., Stear, M. J., Irvine, J., Dick, A., Wallace, D. S., and McKellar, Q. A. (1998). Changes in the zymogenic cell populations of the abomasa of sheep infected with *Haemonchus contortus*. *Parasitology* **116 (Pt 6)**, 569-577.

Scott, I., Dick, A., Irvine, J., Stear, M. J., and McKellar, Q. A. (1999). The distribution of pepsinogen within the abomasa of cattle and sheep infected with *Ostertagia* spp. and sheep infected with *Haemonchus contortus*. *Vet.Parasitol.* **82**, 145-159.

Shaw, R. J., Grimmett, D. J., Donaghy, M. J., Gatehouse, T. K., Shirer, C. L., and Douch, P. G. (1996). Production and characterisation of monoclonal antibodies recognising ovine IgE. *Vet.Immunol.Immunopathol.* **51**, 235-251.

Shaw, R. J., Gatehouse, T. K., and McNeill, M. M. (1998). Serum IgE responses during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int.J.Parasitol.* **28**, 293-302.

Shoop, W. L., Haines, H. W., Michael, B. F., and Eary, C. H. (1993) Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. *Vet.Record*, **133(18)**, 445-7.

Simpson, H. V., Lawton, D. E., Simcock, D. C., Reynolds, G. W., and Pomroy, W. E. (1997). Effects of adult and larval *Haemonchus contortus* on abomasal secretion. *Int.J.Parasitol.* **27**, 825-831.

Smith, T. S., Graham, M., Munn, E. A., Newton, S. E., Knox, D. P., Coadwell, W. J., McMichael-Phillips, D., Smith, H., Smith, W. D., and Oliver, J. J. (1997). Cloning and characterization of a microsomal aminopeptidase from the intestine of the nematode *Haemonchus contortus*. *Biochim.Biophys.Acta* **1338**, 295-306.

Smith, W. D. (1999). Prospects for vaccines of helminth parasites of grazing ruminants. *Int.J.Parasitol.*, **29**, 17-24.

Smith, W. D., Jackson, F., Jackson, E., and Williams, J. (1983). Local immunity and *Ostertagia circumcincta*: changes in the gastric lymph of immune sheep after a challenge infection. *J.Comp Pathol.* **93**, 479-488.

Smith, W. D., Jackson, F., Jackson, E., Williams, J., Willadsen, S. M., and Fehilly, C. B. (1984a). Resistance to *Haemonchus contortus* transferred between genetically histocompatible sheep by immune lymphocytes. *Res.Vet.Sci.* **37**, 199-204.

Smith, W. D., Jackson, F., Jackson, E., Williams, J., and Miller, H. R. (1984b). Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. *J.Comp Pathol.* **94**, 591-601.

Smith, W. D., Jackson, F., Jackson, E., and Williams, J. (1985). Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2- and 10-month-old lambs. *J.Comp Pathol.* **95**, 235-245.

Smith, W. D., Jackson, F., Jackson, E., Graham, R., Williams, J., Willadsen, S. M., and Fehilly, C. B. (1986). Transfer of immunity to *Ostertagia circumcincta* and IgA memory between identical sheep by lymphocytes collected from gastric lymph. *Res.Vet.Sci.* **41** , 300-306.

Smith, W. D. and Smith, S. K. (1993). Evaluation of aspects of the protection afforded to sheep immunised with a gut membrane protein of *Haemonchus contortus*. *Res.Vet.Sci.* **55**, 1-9.

Smith, W. D., Smith, S. K., and Murray, J. M. (1994). Protection studies with integral membrane fractions of *Haemonchus contortus*. *Parasite Immunol.* **16**, 231-241.

Soulsby, E. J. (1985). Advances in immunoparasitology. *Vet.Parasitol.* **18**, 303-319.

Spedding, C.R.W., Brown, T.H. and Large, R.V. (1963). The effect of milk intake on nematode infestation of the lamb. *Proceedings of the nutrition society*. **22**, 32-41.

Stear, M. J., Bairden, K., Bishop, S. C., Duncan, J. L., Karimi, S. K., McKellar, Q. A., and Murray, M. (1995a). Different patterns of faecal egg output following infection of Scottish Blackface lambs with *Ostertagia circumcincta*. *Vet. Parasitol.* **59**, 29-38.

Stear, M. J., Bishop, S. C., Doligalska, M., Duncan, J. L., Holmes, P. H., Irvine, J., McCririe, L., McKellar, Q. A., Sinski, E., and Murray, M. (1995b). Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. *Parasite Immunology* **17**, 643-652.

Stear, M. J. and Wakelin, D. (1998). Genetic resistance to parasitic infection. *Rev.sci.tech* **17**, 143-153.

Stear, M. J., Strain, S., and Bishop, S. C. (1999). Mechanisms underlying resistance to nematode infection. *Int.J.Parasitol.* **29**, 51-56.

Stear, M. J., Henderson, N. G., Kerr, A., McKellar, Q. A., Mitchell, S., Seeley, C., and Bishop, S. C. (2002). Eosinophilia as a marker of resistance to *Teladorsagia circumcincta* in Scottish Blackface lambs. *Parasitology* **124**, 553-560.

Steel, J. W., Jones, W. O., and Wagland, B. M. (1990) The response of immune sheep to challenge with *Trichostrongylus colubriformis*: enteric plasma loss and secretion of biogenic amines. *Int.J.Parasito.*, **20 (8),**1067-73.

Stevenson, L. M., Huntley, J. F., Smith, W. D., and Jones, D. G. (1994). Local eosinophil- and mast cell-related responses in abomasal nematode infec. *Immunology*. **82**, 167-73.

Stewart, D.F. (1953). Studies on resistance of sheep to infestation with *Haemonchus contortus* and *Trichostrongylus* spp. and on the immunological reactions of sheep exposed to infestation. V. The nature of the "self-cure" phenomenon. *Australian Journal of Agricultural Research*, **4**, 100-117.

Stewart, D.F. (1955). "Self-cure" in nematode infestations of sheep. *Nature (London)*, **176**, 1273-1274.

Strain, S. A., Bishop, S. C., Henderson, N. G., Kerr, A., McKellar, Q. A., Mitchell, S., and Stear, M. J. (2002). The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. *Parasitology* **124**, 545-552.

Tabouret, G., Lacroux, C., Andreoletti, O., Bergeaud, J. P., Hailu-Tolosa, Y., Hoste, H., Prevot, F., Grisez, C., Dorchies, P., and Jacquiet, P. (2003). Cellular and humoral local immune responses in sheep experimentally infected with *Oestrus ovis* (Diptera: Oestridae). *Vet.Res.* **34**, 231-241.

Taylor, M. A. and Hunt, K. R. (1989). Anthelmintic drug resistance in the UK. *Vet.Rec.* **125**, 143-147.

Thatcher, E. F. and Gershwin, L. J. (1988). Generation and characterization of murine monoclonal antibodies specific for bovine immunoglobulin E. *Vet.Immunol.Immunopathol.* **18**, 53-66.

Thomas, R.J and Stevens, A.J. (1956). Some observations on *Nematodirus* disease in Northumberland and Durham. *Vet.Record.* **68**, 471-475.

Urquhart, G. M., Jarrett, W. F., Jennings, F. W., McIntyre, W. I., and Mulligan, W. (1966). Immunity to *Haemonchus contortus* infection: relationship between age and successful vaccination with irradiated larvae. *Am.J.Vet.Res.* **27**, 1645-1648.

Urquhart, G. M. (1990). Parasitism in young animals. *Ann.Parasitol.Hum.Comp* **65 Suppl 1**, 77-78.

Urquhart, G.M., Armour, J., Duncan, A.M., Jennings, F.W., Editors (1991). *Vet. Parasitol.*, Longman Scientific & Technical, UK. (Chapter 1).

van den Broek, A. H., Huntley, J. F., Halliwell, R. E., Machell, J., Taylor, M., and Miller, H. R. (2003). Cutaneous hypersensitivity reactions to *Psoroptes ovis* and Der p 1 in sheep previously infested with P. ovis-the sheep scab mite. *Vet.Immunol.Immunopathol.* **91**, 105-117.

van Houtert, M. F., Barger, I. A., Steel, J. W., Windon, R. G., and Emery, D. L. (1995). Effects of dietary protein intake on responses of young sheep to infection with *Trichostrongylus colubriformis*. *Vet.Parasitol.* **56**, 163-180.

van, Die., I, Gomord, V., Kooyman, F. N., van den Berg, T. K., Cummings, R. D., and Vervelde, L. (1999) Core alpha1-->3-fucose is a common modification of N-glycans in parasitic helminths and constitutes an important epitope for IgE from *Haemonchus contortus* infected sheep. *FEBS letter*, **463 (1-2)**, 189-93.

Verwaerde, C., Joseph, M., Capron, M., Pierce, R. J., Damonneville, M., Velge, F., Auriault, C., and Capron, A. (1987) Functional properties of a rat monoclonal IgE antibody specific for *Schistosoma mansoni*. *Journal of Immunology*, **138 (12)**, 4441-6.

Wakelin, D. (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**, 515-524.

Wakelin, D. and Lloyd, M. (1976). Accelerated expulsion of adult *Trichinella spiralis* in mice given lymphoid cells and serum from infected donors. *Parasitology* **72**, 307-315.

Wakelin, D. (1978). Genetic control of susceptibility and resistance to parasitic infection. *Adv.Parasitol.* **16**, 219-308.

Waller, P. J. (1997). Nematode parasite control of livestock in the tropics/subtropics: the need for novel approaches. *Int.J.Parasitol.* **27**, 1193-1201.

Walsh, G. M. (1999). Advances in the immunobiology of eosinophils and their role in disease. *Crit Rev.Clin.Lab Sci.* **36**, 453-496.

Watson, D. L. and Gill, H. S. (1991). Effect of weaning on antibody responses and nematode parasitism in Merino lambs. *Res.Vet.Sci.* **51**, 128-132.

Weiss, R. A., Chanana, A. D., and Joel, D. D. (1986) Postnatal maturation of pulmonary antimicrobial defense mechanisms in conventional and germ-free lambs. *Pediatr. Res.***20 (6)**, 496-504.

Willadsen, P., Riding, G. A., McKenna, R. V., Kemp, D. H., Tellam, R. L., Nielsen, J. N., Lahnstein, J., Cobon, G. S., and Gough, J. M. (1989). Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. *J.Immunol.* **143**, 1346-1351.

Williamson, J.F., Blair, H.T., Garrick, D.J., Pomroy W.E., and Douch, P.G.C (1994). The relationship between internal parasite burden, faecal egg count, and mucosal mast cells in fleeceweight-selected and control sheep. *Proceedings of the New Zealand Society of animal production* **54**, 9-13.

Windon, R. G. and Dineen, J. K. (1980). The segregation of lambs into responders and non-responders: Response to vaccination with irradiated *Trichostrongylus colubriformis* larvae before weaning. *Int.J.Parasitol.*, **10**, 65-73.

Windon, R. G. (1990). Selective Breeding for the control of nematodiasis in sheep. *Rev.sci.tech* **9**, 555-576.

Windon, R. G., Dineen, J. K., Gregg, P., Griffiths, D. A., and Donald, A. D. (1984). The role of thresholds in the response of lambs to vaccination with irradiated *Trichostrongylus colubriformis* larvae. *Int.J.Parasitol.* **14**, 423-428.

Windon, R. G. (1996). Genetic control of resistance to helminths in sheep. *Vet.Immunol.Immunopathol.* **54**, 245-254.

Winter, M. D., Wright, C., and Lee, D. L. (1997) The mast cell and eosinophil response of young lambs to a primary infection with *Nematodirus battus*. *Parasitology*, **114**, 189-93.

Winter, M. D. (2002). *Nematodirus battus* 50 years on--a realistic vaccine candidate? *Trends Parasitol.* **18**, 298-301.

Wolstrup, J., Gronvold, J., Henriksen, S. A., Nansen, P., Larsen, M., Bogh, H. O., and Isloe, B. (1994). An attempt to implement the nematode-trapping fungus *Duddingtonia flagrans* in biological control of trichostrongyle infections of first year grazing calves. *Journal of Helminthology* **68**, 175-180.

Woodbury, R. G., Miller, H. R., Huntley, J. F., Newlands, G. F., Palliser, A. C., and Wakelin, D. (1984). Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. *Nature* **312**, 450-452.

Woolaston, R. R., Barger, I. A., and Piper, L. R. (1990). Response to helminth infection of sheep selected for resistance to *Haemonchus contortus*. *Int.J.Parasitol.*, **20**, 1015-1018.

Woolaston, R. R. and Baker, N. F. (1996). Prospects of breeding small ruminants for resistance to internal parasites. *Int.J.Parasitol.*, **26**, 845-855.

Woolaston, R. R. (1992). Selection of Merino sheep for increased and decreased resistance to *Haemonchus contortus*: peri-parturient effects on faecal egg counts. *Int.J.Parasitol.* **22 (7)**, 947-53.

Yadav, C. L., Grewal, H. S., and Banerjee, D. P. (1993). Susceptibility of two crossbreeds of sheep to *Haemonchus contortus*. *Int.J.Parasitol.* **23**, 819-822.

Yahiro, S., Cain, G., and Butler, J. E. (1998). Identification, characterization and expression of *Toxocara canis* nematode polyprotein allergen TBA-1. *Parasite Immunol.* **20**, 351-357.

Appendices

0.1M Carbonate coating Buffer, pH 9.6 for ELISAs

Solution A: - 0.2M Anhydrous Sodium Carbonate (Na_2Co_3)

Na_2Co_3	21.2g
Distilled Water (dH_2O)	1000ml

Solution B: - 0.2M Sodium Hydrogen Carbonate ($NaHCO_3$)

$NaHCO_3$	16.8g
dH_2O	1000ml

To prepare the carbonate coating buffer solutions: -

Solution A	80ml
Solution B	170ml
dH_2O	750ml

Adjust pH to 9.6.

PBS T20

10X Phosphate buffered saline (PBS)	500ml
dH_2O	4500ml
Polyoxyethylene-sorbitan monolaurate (Tween 20), SIGMA code P-7949	2.5ml

PBS Tween 80 + Sodium Chloride (NaCl)

1X PBS	10L
NaCl	292.2g
Polyoxyethylene-sorbitan monooleate (Tween 80), SIGMA code P-8074	50ml

Carpentiers reagent A

Eosin Y	0.5g

Add 20ml dH_2O. Dissolve, and then make up to 25ml with dH_2O. Store at room temperature.

Carpentiers reagent B

Calcium carbonate	0.5g
Formalin (37% formaldehyde)	100ml

Mix together in fume cupboard. Store at room temperature.

Carpentiers stain

Carpentiers reagent A	2ml
Carpentiers reagent B	3ml

Mix reagents together and make up to 100ml with dH_2O. Store at 4°C up to 1 week.

Resolving gel Buffer for SDS-PAGE gels

Tris	60.5g
dH_2O	500ml

Add concentrated hydrochloric acid to bring pH down to 8.8 (~ 5ml).

Stacking gel buffer for SDS-PAGE gels

Tris	30.25g
dH_2O	500ml

Add concentrated HCL to bring pH down to 6.8 (~20ml)

20% SDS solution

SDS	40g
dH_2O	200ml

10% Ammonium Persulphate (APS)

APS	1g
dH_2O	10ml

Non-Reducing Sample Buffer for SDS-PAGE gels

Stacking Buffer	2.5ml
dH_2O	2.3ml
Glycerol	2.0ml
20% SDS	2.0ml
0.1% Bromophenol Blue (SIGMA B 7021)	0.2ml

Reducing Sample Buffer for SDS-PAGE gels

Stacking Buffer	2.5ml
dH_2O	2.3ml
Glycerol	2.0ml
20% SDS	2.0ml
β - Mercaptoethanol (SIGMA M 6250)	1.0ml
0.1% Bromophenol Blue (SIGMA B 7021)	0.2ml

Running Buffer 5X for SDS-PAGE gels

Tris	45g
Glycine	216g
SDS (Lauryl Sulphate)	15g
dH_2O	3L

Transfer Buffer for western blot analysis

TRIZMA Base (TRIS)	5.8g
Glycine	2.9g
SDS	0.37g
Methanol	200ml
dH_2O	800ml

Add together the above reagents in a 1-litre Duran bottle. Mix well.

WesternBreeze® Chemiluminescent Kit: -

Blocking Solution

Blocker/Diluent Part A (concentrated buffered saline solution containing detergent)	4ml
Blocker/Diluent Part B (concentrated Hammerstein casein solution)	2ml
dH_2O	14ml

Add Part A to distilled water and mix. Then add Part B.

Secondary antibody diluent

As above.

Wash solution

Wash Solution (concentrated buffered saline solution containing detergent)	10ml
dH_2O	150ml

Tertiary Antibody Solution

Ready-to-use solution of alkaline phosphatase-conjugated, affinity purified, anti-species IgG.

Chemiluminescent Substrate

Ready-to-use solution of CDP-Star® chemiluminescent substrate for alkaline phosphatase.

Chemiluminescent Substrate Enhancer

Nitro-Block-II™ enhancer (trademark of Tropix, Inc.).

3% Acetic acid

Glacial acetic acid	30ml
dH_2O	970ml

Store at room temperature.

50% Methanol

Methanol	250ml
dH_2O	250ml

Store at room temperature

Oxidation Solution for glycoprotein kit

Add 250ml 3% acetic acid to the bottle labelled "Oxidation Reagent" and mix until material is completely dissolved. Store the solution at room temperature.

Reduction Solution for glycoprotein kit

Add 250ml dH_2O to the bottle labelled "Reduction Reagent" and mix until material is completely dissolved. Store the solution at room temperature.

Horseradish Peroxidase Positive Control for glycoprotein kit

Reconstitute contents of vial with 0.5ml of dH_2O to produce a 2mg/ml solution. Dilute to a 1mg/ml solution with the sample buffer for SDS-PAGE analysis. Aliquot and store at –20°C.

Soybean Trypsin Inhibitor Negative Control for glycoprotein kit

Reconstitute contents of vial with 0.5ml of dH_2O to produce a 2mg/ml solution. Dilute to a 1mg/ml solution with the sample buffer for SDS-PAGE analysis. Aliquot and store at –20°C.

Proceedings of Meetings

10th ICOPA, Vancouver, Canada, August 2002.

Pettit,J.J, Huntley, J.F, Jackson, F, Rocchi, M. *Immunological markers associated with responsiveness against Teladorsagia infections in lambs*.p208 PA-085

Novel Approaches to Helminth Control III, Edinburgh, UK, July 2002.

Pettit,J.J, Huntley, J.F, Jackson, F, Rocchi, M. *The relationship between responsiveness against gastrointestinal nematodes in lambs and the numbers of circulating IgE bearing cells*.p12

World Association for the Advancement of Veterinary Parasitology Stresa, Italy, August 2001.

Pettit,J.J, Huntley, J.F, Jackson, F (WAAVP), *The use of immunoglobulins as markers for responsiveness against Teladorsagia infections in sheep*.p74, F4